CELLULAR & MOLECULAR MECHANISMS THAT CONTRIBUTE TO THE EARLY DEVELOPMENT OF SKELETAL MUSCLE & SYSTEMIC INSULIN RESISTANCE

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DEDICATION

I would like to dedicate this dissertation especially to Robert and Laura Grice, my parents. To my siblings and their children Robert Grice Jr., James and Annistyn Grice, Erin, Adam, Ethan, Logan, and Katelynn Wartsbaugh. And mostly, my soon to be wife Elizabeth Cozzi (soon to be Grice) and our future family. These people, and the future I will spend with them, has been an amazing motivating force in my life. My parents were wonderful hardworking people who sacrificed a ton to raise their children well and are always available when I need them the most. My siblings, nieces, and nephews were always a reliable source of fun and entertainment when I needed downtime. Elizabeth has been a solid friend in times of need and has been with me for the tough times in medical and graduate school. Our future together has been the single most motivating factor in times of challenges and adversity.

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Brian A. Grice

CELLULAR & MOLECULAR MECHANISMS THAT CONTRIBUTE TO THE EARLY DEVELOPMENT OF SKELETAL MUSCLE & SYSTEMIC INSULIN RESISTANCE

Insulin resistance starts years before type 2 diabetes (T2D) diagnosis, even before recognition of prediabetes. Mice on a high fat diet have a similar early onset of insulin resistance, yet the mechanism remains unknown. Several studies have demonstrated that skeletal muscle insulin resistance resulting from obesity or high fat feeding does not stem from defects in proximal insulin signaling. Our lab discovered that excess plasma membrane cholesterol impairs insulin action. Excess cholesterol in the plasma membrane causes a loss of cortical actin filaments that are essential for glucose transporter GLUT4 regulation by insulin. Our cell studies further revealed that increased hexosamine biosynthesis pathway (HBP) activity increases O-linked N-acetylglucosamine modification of the transcription factor Sp1, leading to transcription of HMG-CoA reductase (HMGR), the rate-limiting enzyme in cholesterol biosynthesis. Our central hypothesis is that cholesterol accumulation mediated by HBP activity is an early reversible mechanism of high-fat diet-induced insulin resistance. We performed a series of studies and found that early high-fat feeding-induced insulin resistance is associated with a buildup of cholesterol in skeletal muscle membranes (SMM). Akin to the antidiabetic effect of caloric restriction, we found that high-fat diet removal fully mitigated SMM cholesterol accumulation and insulin resistance. Furthermore, using the cholesterol-binding agent methyl-β-cyclodextrin (MβCD), studies established causality between excess SMM cholesterol and insulin resistance. To begin to assess the role of the

HBP/Sp1 in contributing to de novo cholesterol biosynthesis, SMM accumulation, and insulin resistance we treated high-fat fed mice with an Sp1 inhibitor, mithramycin. We found that mithramycin prevented SMM cholesterol accumulation and insulin resistance. This series of studies provide evidence that HBP/Sp1-mediated cholesterol accumulation in SMM is a causal, early and reversible mechanism of whole body insulin resistance.

Jeffrey Elmendorf, Ph.D., Chair

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Chapter 1. Introduction

1.A. Epidemiology of type 2 diabetes and insulin resistance

The global prevalence of diabetes mellitus is estimated to be 422 million resulting in 1.5 million early deaths a year[1]. The economic cost of diabetes for the United States was an estimated 327 billion dollars in 2017 a 26% increase from just 5 years prior[2]. Diabetes is concerning since it affects almost all parts of the body and causes serious health issues, including being the leading cause of retinopathy, kidney failure, and amputations in the United States. Moreover, diabetes augments the risk of heart attack, stroke, high blood pressure, high triglycerides, high cholesterol, and infections.

Type 2 diabetes is the fastest growing disease and is the most prevalent form of diabetes mellitus worldwide[1]. Insulin resistance, the defining feature of type 2 diabetes, is the inability of insulin to effectively decrease blood glucose levels. Insulin resistance occurs many years prior to the onset of type 2 diabetes, and for a sizeable percentage (33.9%) of the adult population in the United States of America, insulin resistance can be clinically identified by the presence of impaired glucose regulation (IGR), or prediabetes[3]. This population is at an elevated risk for type 2 diabetes and other metabolic diseases.

Clinically, insulin resistance can be identified by the presence of IGR defined as either impaired fasting glucose (IFG) or impaired glucose tolerance (IGT)[4]. About 40% of people with IGR display IFG, a marker of hepatic insulin resistance[5]. A similar percentage of IGR individuals display IGT resultant from skeletal muscle insulin resistance[5]. Incident cases of type 2 diabetes develop from skeletal muscle or hepatic insulin resistance alone, rarely both[5]. Only in people with combined IFG/IGT (~16%) is both hepatic and skeletal muscle resistance present[4-9].

Blood glucose homeostasis is maintained predominately by the liver, skeletal muscle, and fat. Fasting blood glucose is maintained at a stable level by hepatic glucose production through glycogenolysis and gluconeogenesis. Hepatic insulin resistance is the inability of insulin to suppress hepatic glucose output resulting in elevated fasting glucose[10]. Once absorbed by the gut, glucose stimulates the secretion of insulin by pancreatic β-cells. Post-prandial insulin secretion inhibits hepatic glucose output and activates glucose disposal in skeletal muscle, and to a lesser extent adipose tissue. Skeletal muscle utilizes glycogen for energy during fasting while glucose disposal is minimal. Post-prandial insulin secretion, however, upregulates skeletal muscle glucose disposal and glycogen synthesis, eventually lowering blood glucose to basal levels by storing glucose as glycogen. Skeletal muscle insulin resistance is the inability of insulin to sufficiently stimulate glucose disposal resulting in elevated post-prandial blood glucose[10, 11].

Fat accounts for a small percentage of insulin-stimulated glucose disposal and therefore, similarly has diminished glucose disposal[11]. However, the major response of fat to insulin is the inhibition of lipolysis and the activation of lipogenesis for fuel storage. The effect of fat on blood glucose is mainly through the augmentation of hepatic gluconeogenesis which will be discussed later[10, 11].

Richard Bergman in the 1980s modeled the relationship between insulin resistance and insulin secretion adding to our understanding of IGR[12]. The modeling identified a log-linear relationship between insulin secretion (AIR_G) and insulin sensitivity (S_I)[12]. Understanding this relationship determines what Bergman termed the disposition index ($DI=AIR_G \times S_I$) and it first became apparent that hyperinsulinemia can

maintain normoglycemia in the presence of insulin resistance[12, 13]. It is estimated that a quarter of non-obese normoglycemic individuals have some form of insulin resistance[14]. The transition from insulin resistance to IGR and type 2 diabetes occurs after pancreatic β cells fail to secrete insulin at quantities sufficient to compensate for insulin resistance[14].

In 2001 the diabetes prevention program discovered that type 2 diabetes can be prevented in people with IGT. Prevention was successful with either a lifestyle intervention or metformin therapy, some individuals even reverted to normal glucose tolerance[15, 16]. The onset of IGT, however, may be too late for many people. As seen in prospective studies, insulin secretion begins to diminish for many at the onset of IGT as β-cell failure culminates[17]. Therefore, identifying the pathophysiology of skeletal muscle insulin resistance well before pancreatic β cells are damaged would be of tremendous value for the intervention of type 2 diabetes.

1.B. Physiology of insulin resistance

A decade following the discovery of insulin was the characterization of insulin resistance by Himsworth[18]. In 1959, Yalow and Berson, following their development of the radioimmunoassay, investigated the endogenous insulin levels in the blood of insulin-resistant and insulin-sensitive populations[19, 20]. Their research led to the discovery that glucose-intolerant subjects had hyperinsulinemia and hyperglycemia[20]. Shen and colleagues helped further validate the presence of insulin resistance in 1970 by maintaining steady glucose concentrations in the presence of insulin, epinephrine and propranolol to inhibit insulin secretion and hepatic glucose production[21]. They found that higher steady state concentrations of glucose corresponded to impaired skeletal muscle glucose disposal. In 1979, DeFronzo, Andres and Tobin, developed the euglycemic

clamp which has since become the gold standard for identifying insulin resistance[22]. The clamp has also allowed scientists to distinguish between hepatic insulin resistance and skeletal muscle insulin resistance. The relationship mentioned in the previous section between IFG and IGT with hepatic and skeletal muscle insulin resistance, respectively, was understood using the clamp method[4, 6, 7].

Skeletal muscle is the site responsible for nearly 85% of whole body glucose disposal making it an important mediator of human glucose tolerance[23]. Clamp measured skeletal muscle insulin sensitivity begins to decline quickly in the setting of positive energy balance prior to any observed clinical indicators[7, 13, 24, 25]. Notably, individuals with normal glucose tolerance, but a low level of glucose disposal are at an elevated risk for IGT, regardless of insulin secretory function[26]. On the other hand, it has recently been observed that hyperinsulinemia is an independent mediator of insulin resistance irrespective of base-line insulin sensitivity[27]. The inability of insulin to fully stimulate the insulin responsive glucose transporter type 4 (GLUT4) translocation to the cell-surface membrane accounts for diminished glucose uptake[28]. Metabolism of glucose, glycogen, proteins, and fat are altered in skeletal muscle insulin resistance, yet these metabolic changes are modest and secondary to the defining characteristic of skeletal muscle insulin resistance, namely impaired GLUT4 regulation[10, 11, 29].

Insulin also regulates anabolic and catabolic functions in fat and the liver. As mentioned earlier the main node of insulin regulation in fat is lipogenesis and lipolysis. Lipolysis in fat is no longer attenuated by insulin and consequently non-esterified fatty acid (NEFA) levels increase in the blood[10]. Hepatic insulin resistance corresponds to an increased activation of glycogenolysis and gluconeogenesis. This increase is partly

mediated by insulin's inability to allosterically regulate glycogen synthase, and the transcriptional regulation of gluconeogenic genes (Pck1 and G6pc)[29]. Proteolysis and hepatic lipolysis are upregulated providing fuel for gluconeogenesis, but these hepatic processes appear to be minor contributors to gluconeogenesis[10]. Instead, hepatic insulin resistance appears to be tightly coupled to the increased NEFA levels that arise from the insulin resistance of fat[29, 30]. Increasing the concentration of NEFAs in circulation is sufficient to induce hepatic glucose production during a clamp in insulin sensitive subjects[10]. The liver also has a pathway-specific resistance since lipogenesis remains functional, or even augmented. My dissertation is focused on skeletal muscle insulin resistance and I would therefore, like to direct the reader to the review articles by Czech, Hatting et al., and Petersen et al. as guides to better understand the physiology of fat and liver insulin resistance[10, 29, 31].

1.C. Regulation of GLUT4 translocation

While insulin resistance was just beginning to be appreciated and the physiology was under study, Rachmiel Levine, in 1950, observed insulin's ability to regulate glucose uptake in muscle[32]. The ensuing discovery of the membrane-bound insulin receptor, in 1971, led investigators to probe insulin signal transduction[33]. Throughout the 1980s it became apparent that insulin was regulating glucose uptake in fat and skeletal muscle by relocation of a glucose transporter from the cytosol to the plasma membrane[34-45]. By 1988, David James identified an insulin-regulated glucose transporter unique to striated muscle and fat, which we now know as GLUT4[37]. A year later the gene encoding GLUT4 was cloned and mapped[36]. Since then, scientists have identified many of the effectors in the insulin signal transduction pathway and the response elements regulating the

translocation of GLUT4 to the plasma membrane[10, 45-48]. The following section reviews the intricate pathway of insulin-regulated GLUT4 translocation (see Figure 1 for graphical depiction). Many of the signaling proteins in this pathway have multiple isoforms and phosphorylation sites, but I will only discuss those responsible for insulin-regulated GLUT4 translocation. I will first discuss the proximal signal transduction and then review the mechanical processes executed by the proximal signal. Expanded information on the biochemistry of the insulin receptor and its signaling pathways can be found in the review articles by Haeusler, Hoffman, Jaldin-Fincat, Leto, Petersen, and Tokarz[10, 45, 46, 48].

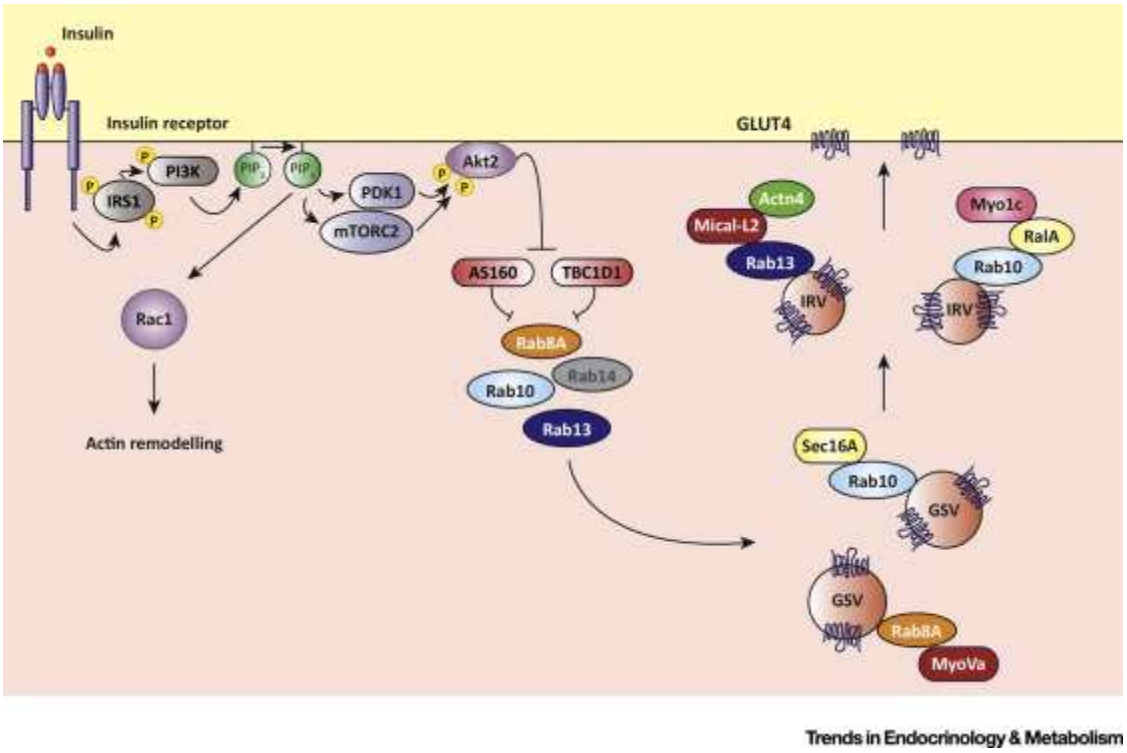


Figure 1. Insulin regulated GLUT4 translocation. The sequence of signaling steps is as follows: 1) Insulin binds the insulin receptor 2) insulin receptor auto-phosphorylates 3) insulin receptor phosphorylates IRS 4) IRS activates PI3K by docking to SH domain 5) PI3K converts PIP2 to PIP3 6) PIP3 activates PDK1 and mTORC2 by recruiting them to the plasma membrane through PH-domains 7) PDK1 and mTORC2 phosphorylate Akt2 8) Akt2 phosphorylates AS160 releasing it from inhibitory binding Rabs. 9) Rabs recruit GSVs to docking stations and interact with fusion proteins resulting in GLUT4 integration into the plasma membrane. Figure adapted from Jaldin-Fincat et al.[46]

1.C.1. Proximal insulin signaling

There are two insulin receptor isoforms A and B, the isoform most sensitive to insulin and expressed in large quantity on insulin responsive tissues is insulin receptor B[10]. This tyrosine kinase receptor undergoes autophosphorylation in response to insulin binding[49]. The insulin receptor produces a mitogenic signal through a mitogen-activated protein kinase and a metabolic signal through an insulin receptor substrate (IRS) of which there are six isoforms IRS1, IRS2, IRS3, IRS4, IRS5, and IRS6[48, 50]. IRS1 amplifies the insulin signal by effector signal propagation and is necessary for GLUT4 translocation whereas IRS2 is involved in other cellular metabolic functions and, along with IRS1, regulates hepatic insulin signaling[10]. The importance of IRS3-6 are imperfectly understood.

The insulin receptor phosphorylates multiple tyrosine residues in IRS1[50]. Once phosphorylated, IRS1 docks to the regulatory subunit, p85, of phosphatidylinositol 3 kinase 3(PI3K) which phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) generating phosphatidylinositol 3,4,5-bisphosphate (PIP3). The PIP3 signal transduction regulating GLUT4 translocation is PI3K-dependent and the nonspecific PI3K inhibitor wortmannin can effectively eliminate skeletal muscle glucose uptake[10]. Studies in 3T3-L1 adipocytes, however, show that insulin can regulate GLUT4 translocation independent of PI3K, and there is emerging evidence that skeletal muscle also has a PI3K-independent pathway not discussed[10]. Once activated PIP3 transmits the signal to Akt2, RAC1, and Protein Kinase C (PKC) three divergent pathways each important for GLUT4 translocation[10, 46-48, 51].

1.C.2. GLUT4 translocation

The first PIP3 responsive pathway identified was the transmission of the insulin signal from PIP3 to phosphoinositide-dependent protein kinase 1 (PDK1) and mTORC2[46]. PDK1 and mTORC2 are kinases responsible for the phosphorylation of Akt2—the isoform of Akt responsible for regulating GLUT4 translocation—on a threonine residue and a serine residue, respectively[10, 46]. The PDK1 phosphorylation of the threonine site occurs first and is required for GLUT4 translocation[10, 46, 52]. The serine residue is phosphorylated second and is not necessary for GLUT4 translocation[10, 46]. Instead, it is responsible for regulating the Akt2-mediated transcriptional response[10]. Activation of Akt at the threonine residue results in the phosphorylation of an Akt substrate of 160 kD (AS160, aka TBC1D4) and another isoform TBC1D1, rendering these Akt targets inactive[10]. AS160 and TBC1D1 are Rab GTPase-activating protein (GAP)s that inhibit Rab8A and Rab13 in skeletal muscle and Rab10 in adipose tissue[10, 45, 46, 53]. Liberation of these Rabs from the GAP proteins allow GLUT4 storage vesicle (GSV)s to traffic to the plasma membrane[10, 45, 46, 53].

The second pathway regulated by PIP3 is responsible for assembling the cortical actin that facilitates tethering, docking, and fusion of GSVs to the plasma membrane. PIP3 activates Rac1 which is responsible for inhibiting the actin disassembly protein Cofilin and activating the actin-branching protein Arp2/3[10, 45, 46, 53]. Akt2 also mediates the stabilization of actin at the membrane once it is assembled. PIP2 also acts in a PI3K-independent capacity by regulating filamentous actin (F-actin) at the plasma membrane. The motor proteins MyoVa and Mical-L2 interact with the actin transporting the GSV to the point of tethering[10, 45, 46, 53].

The third point of PI3K signal regulation is located at the membrane and is facilitated by PKCζ and PKCλ. Once triggered by phosphorylation, these PKCs phosphorylate Munc18c releasing it from the inhibitory binding of the docking/fusion protein Syntaxin4[10, 45, 46, 53]. At the convergence of the GSVs with the plasma membrane the pathways reconvene to complete translocation[10, 45, 46, 53]. Once situated, the Rabs, activated by GAP inhibition, can promote the tethering of vesicle membrane protein Vamp2 to the plasma membrane protein SNAP23 [10, 45, 46, 53]. PI3K signaling to phospholipase D1 (PLD1) results in phosphatidylcholine conversion to phosphatidic acid. This conversion induces a negative curvature in the plasma membrane, which is a "priming signal" for fusion[45].

There are many points susceptible to disruption along the pathway delineated. Knockout studies of proximal signaling proteins point to multiple sites that can cause insulin resistance in homozygous knockouts. Heterozygous knockout studies complicate this picture and question the causality of proximal signaling in insulin resistance. Knock-in studies further muddy the waters by demonstrating redundancies in the pathway and presenting us with evidence of multiple ancillary pathways capable of bypassing signaling steps. Allosteric inhibition occurs at multiple locations along the proximal signaling pathway and can be induced by cellular and physiologic mediators of insulin resistance. Differential regulation of gene transcription accompanies insulin resistance and may also mediate the effect of diet-induced insulin resistance. GLUT4 translocation has been explored less in diet-induced insulin resistance studies, but evidence from proximal studies and translocation experiments suggest a key role for this process in diet-induced insulin resistance. Our lab has provided a portfolio of evidence that the

mediation of diet-induced insulin resistance occurs by a disruption in the translocation processes at the plasma membrane.

1.D. Cellular mechanisms of insulin resistance

In 1963 Randle and colleagues theorized competition between glucose and fatty acids, or glucose-fatty acid cycle, regulates insulin action either at the cellular membrane or by regulating hexokinase—the enzyme responsible for converting glucose to glucose-6-phosphate (G6P)—the first step in glycolysis[54]. The contention was that unregulated adipose lipolysis in insulin resistance saturates the skeletal muscle with NEFAs. The muscle oxidizes NEFAs converting them in a series of steps to malonyl-CoA. Randle postulated malonyl-CoA was responsible for inhibiting glucose uptake through hexokinase inhibition and switching to β-oxidation[54]. Another aspect is the inhibition of pyruvate dehydrogenase in turn elevating levels of pyruvate and lactate which ultimately are shuttled into hepatic gluconeogenesis[54].

The regulation of insulin stimulated glucose uptake has turned out to be more complicated and cannot be explained simply by the glucose-fatty acid cycle. In human studies, the worsening of IGT, a marker of skeletal muscle insulin resistance, can be unrelated to NEFA concentration in the blood. Furthermore, in some populations, NEFAs are inversely correlated with diabetes risk after adjusting for 2-hour plasma glucose[55, 56]. Supporting these findings are the epidemiologic trends I discussed early regarding IGR. In most cases of insulin resistance, increased hepatic glucose output and skeletal muscle insulin resistance are not present simultaneously. The Randle hypothesis necessitates the concordant presentation of elevated hepatic glucose output and skeletal muscle insulin resistance. More studies examining the Randle hypothesis have shown the central tenant,

fatty acids regulating metabolism, is indeed accurate[57]. The contention, however, that fatty acid regulation of glycolysis mediates diet-induced insulin resistance is insufficient in explaining insulin resistance[10, 58]. There is a lot of evidence that hepatic glucose output is partly mediated by NEFA levels in the blood, but skeletal muscle insulin resistance is unlikely a consequence of circulating NEFA levels. And, although it is well known that glycolytic metabolites allosterically regulate glycolytic; gluconeogenic; and glycogenic enzymes, altered allosteric regulation of these pathways is not a major contributor to impaired insulin-stimulated glucose uptake in skeletal muscle[59] and does not account for total hepatic glucose output in insulin resistance[10].

Regulation of metabolic enzymes is mediated in part by insulin regulated transcription factors. Insulin signaling through Akt regulates two important transcription factors fork head family box O 1 (FOXO1) and sterol regulatory binding protein-1C (SREBP-1C)[10, 29]. Akt directly regulates FOXO1 and indirectly mediates SREBP-1C through the mTOR pathway. FOXO1 is responsible for increasing the expression of gluconeogenic proteins, mainly in the liver[48]. Normally inhibited by insulin, FOXO1 becomes unregulated in insulin resistance and this deregulation coupled with elevated circulating NEFAs results in elevated hepatic glucose output[10, 29, 48]. SREBP-1C is required for upregulating fatty acid and cholesterol synthesis in the liver and skeletal muscle. The regulation of these two pathways demonstrates the concept of pathway selective insulin resistance.

Pathway-selective resistance was first coined as a term to describe the conflicting presence of intact insulin receptor-regulated mitogenic activity and metabolic insulin resistance. Now, however, we have many examples of this phenomenon such as tow

conflicting features of hepatic and adipose insulin resistance, 1) FOXO1 upregulation of gluconeogenesis and fat lipolysis are not responsive to Akt regulation, 2) whereas SREBP-1c mediated *de novo* lipogenesis is upregulated by hyperinsulinemic activation of the Akt→mTOR pathway in insulin resistance. Pathway selective insulin resistance has not been identified in skeletal muscle, but this process may explain the presence of unregulated proteolysis and lipolysis in skeletal muscle and the contrary observation that only a small percentage of proximal signaling is required for insulin stimulated GLUT4 translocation[60, 61].

Increased fatty acid synthesis, along with inflammation and mitochondrial dysfunction, endoplasmic reticulum (ER) stress, hyperleptinemia, hyperinsulinemia, and hyperglycemia are all physiologic mediators of insulin resistance. Many of these physiologic processes affect proximal insulin signaling by activation of allosteric regulators. Hyperglycemia and hyperinsulinemia also increase glucose flux through the hexosamine biosynthesis pathway (HBP)—another post-transcriptional regulator of signaling molecules and transcription factors. We and others believe the study of mechanisms arising early in the pathogenesis of insulin resistance will reduce confounding of multiple congruent mechanisms and will be important for revealing the causality of one or more contributing mechanisms[10, 62].

In this section I will provide an overview first of post-translational regulators of insulin signaling molecules, since these are redundant among many of the physiologic mediators of signaling (see Figure 2). I will then discuss the mechanism of distal regulation at the plasma membrane studied as part of my dissertation. Following the discussion of mechanisms, is a presentation of well-known physiologic mediators of

insulin resistance. I will then follow up with a temporal sequence of these mechanisms understood so far for diet-induced insulin resistance. Furthermore, I will discuss the evidence for causality attributed to these mechanisms. I will point to important studies that demonstrate the insufficiency of proximal insulin signaling defects as a mechanism for skeletal muscle diet-induced insulin resistance and direct the reader back to my dissertation subject of membrane cytoskeletal processes.

It is important to note that insulin action in fat, skeletal muscle, and liver has many concordant steps in the insulin-signaling pathway and therefore distinctions will be made when mechanisms diverge and only relate to one or two of these tissues. This overlap is also important when investigating the physiology of insulin resistance because alterations of a common pathway step will likely affect insulin action in all the tissues similarly; whereas, alterations of divergent pathway steps will only affect insulin action in the corresponding tissue.

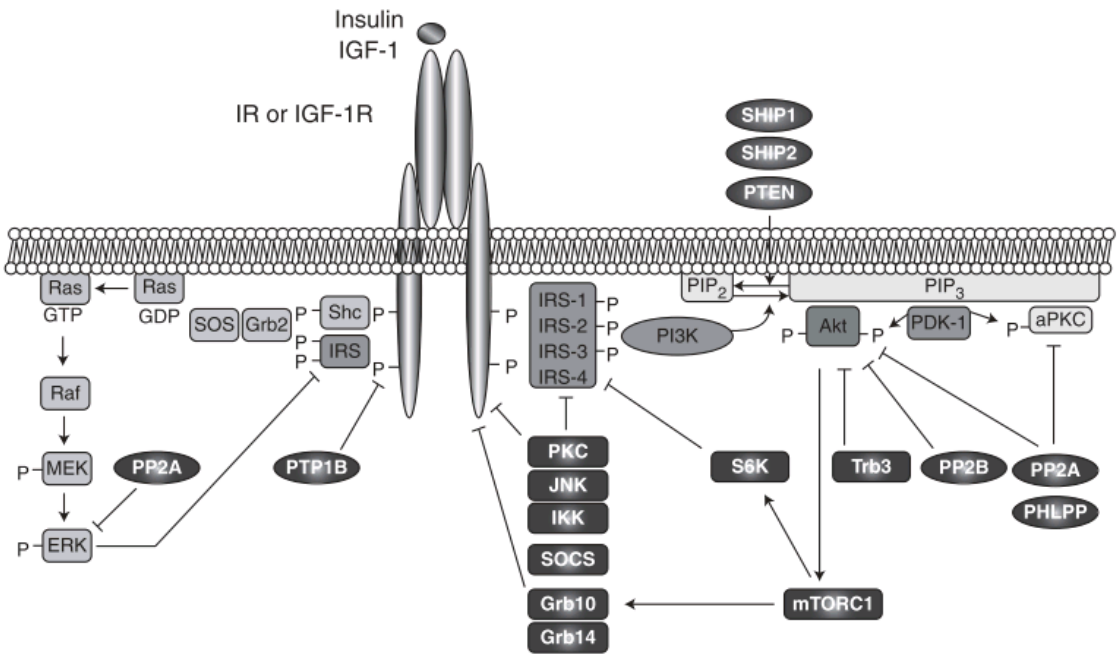


Figure 2. Proximal insulin signaling regulatory proteins. Regulation of insulin signaling pathway is through 5 classes of proteins, protein phosphatases (PTP1B, PP2A, PP2B, PHLPP), lipid phosphatases (SHIP1/2 and PTEN), inhibitory kinases (S6K, aPKCθ/ε, IKKβ, JNK), pseudosubstrates (Grb10, Grb13, Grb14 and SOCs), and protein degradation (clathrin-coated pits, lysosome recruitment, and SOCs activation of ubiquitin-mediated degradation). Most of the known physiologic mediators of insulin resistance mentioned below promote insulin resistance through one of these inhibitory pathways. Figure adapted from Boucher et al.[63].

1.D.1. Post-translational regulation

A well-known form of insulin resistance is caused by a rare mutation in the insulin receptor gene[64]. Individuals with this mutation, present with acanthosis nigricans and severe insulin resistance. This genetic form of insulin resistance is very rare, more common insulin receptor defects associated with insulin resistance are insulin receptor internalization, phosphatase activity, and activity-blocking pseudosubstrates[48, 63].

Hyperinsulinemic activation of the insulin receptor signals to clathrin-coated pits and lysosomes recruitment for receptor internalization, recycling, or degradation[48]. A phosphatase regulating the insulin receptor is protein tyrosine phosphatase 1B (PTP1B) which dephosphorylates tyrosine residues[48, 63]. Homozygous knockout studies of PTP1B improve the insulin sensitivity of mutant mice compared to their wild type counterparts[48]. Pseudosubtrates (Grb10, Grb13, Grb14 and SOCs) once activated, bind to the insulin receptor thereby inhibiting insulin-mediated receptor activation[48, 63]. SOCs also promotes insulin receptor internalization and ubiquitin-mediated degradation[48].

Inhibitory serine/threonine kinases regulate IRS activity by phosphorylating a variety of serine/threonine residues on the insulin receptor and IRS proteins[48]. Well-known inhibitory kinases are atypical protein kinase C (aPKCθ in skeletal muscle; aPKCε in liver), ribosomal protein S6 kinase (S6K), IκB kinaseβ (IKKβ), c-jun N-terminal kinase (JNK), and mTOR. Akt activates mTORC1 creating a negative feedback loop by activating S6K, whereas the other serine/threonine kinases (PKCs, IKKβ, JNK) are activated by intracellular and paracrine signals[48, 63].

PI3K is responsible for the cascading activation of PIP2→PIP3→ PDK1→Akt2/RAC1/PKCζ/ PKCλ and inhibition of PI3K leads to the direct inhibition of

this cascade[10, 45, 65, 66]. PI3K activity is mitigated by lipid phosphatases PTEN and SHIP2 that reverse the action of PI3K converting PIP3→PIP2. PTEN is allosterically regulated by PI3K, such that PI3K activates PTEN leading to feedback inhibition[10, 48, 63]. Direct activation of PKCζ/ PKCλ with okadaic acid can bypass PI3K and stimulate GLUT4 translocation by regulating the cycling of GLUT4 vesicles at the plasma membrane[67].

Regulation of Akt is mainly through the action of protein phosphatases (PP2A, PP2B, PHLPP). Activation of PP2A is modulated by the PP2A phosphatase activator (PTPA) and adenosine triphosphate (ATP)[68]. ATP binds to the catalytic site in PP2A and is required for serine/threonine phosphatase activity[68]. PP2B is regulated by calcium and calmodulin and PHLPP is regulated by magnesium[69, 70]. Along with Akt regulation, PP2A dephosphorylates proteins in mitogenic pathways and PHLPP regulates the activity, and protein concentration, of PKCs[48, 70].

1.D.2. Membrane and cytoskeletal regulation

Unique among the common metabolic functions of insulin action is the mechanical steps required for transporting GSVs from the perinuclear region to the membrane for tethering, docking, and fusion. This process involves a complex coordination of motor proteins, actin remodeling/stabilization proteins, membrane fusion proteins, and the GTPases required to meet energy demands. Disruption in these processes can completely inhibit insulin-stimulated GLUT4 translocation.

Our lab is one of the first groups to have identified these sites as novel locations capable of causing insulin resistance. The many studies published over the past 15 years have shown that disruption of cortical F-actin at the plasma membrane by excess membrane cholesterol impairs insulin stimulated GLUT4 translocation in skeletal

muscle[71, 72]. The PI3K-dependent effector PIP2 was also identified to be an important regulator of plasma membrane F-actin assembly [72, 73]. PIP2 acts at the plasma membrane as a facilitator of GLUT4 translocation through stabilization of F-actin in a manner independent of PI3K[73]. PIP2 stabilizes F-actin at lipid rafts in the plasma membrane, a cholesterol dense region important for endosomal fusion to the plasma membrane[74, 75]. Palmitate decreases PIP2 and F-actin assembly through an unclear mechanism attributed to elevated plasma membrane cholesterol[76]. These cytoskeletal defects were associated with impaired GLUT4 regulation and diminished Akt phosphorylation[77].

A study by Habegger et al. tested the causality of cholesterol-dependent cytoskeletal dysfunction in insulin resistant L6 myotubes by normalization of plasma membrane cholesterol with methyl-β-cyclodextrin[76]. Normalization restored F-actin assembly, GLUT4 translocation, and glucose uptake in insulin-resistant cells. Phospho-Akt2 levels, however, were still impaired indicating this cholesterol-induced cytoskeletal/membrane defect was independent of signaling[77]. Research that followed investigated the physiologic mediator of cholesterol accumulation and revealed a possible cause being increased HBP activity[78]. These studies were the first to indicate the presence of an insulin-signal-independent mediator of lipid-, insulin-, and glucose-induced insulin resistance.

1.D.3. Mediators of insulin resistance

Insulin resistance is an obesity-associated disease that has boomed in communities following the introduction of a high-fat western-style diet[79]. Randle's hypothesis prompted research in the effects of lipids on insulin resistance resulting in many discoveries linking the onset of insulin resistance to lipids. Lipid accumulation in insulin-responsive tissues has been shown to regulate multiple insulin-signaling molecules. Other well-known mediators of insulin resistance are believed to stem, in part, from lipid overload.

Lipid dysregulation by adipose tissue has a profound effect on insulin sensitivity. Adipose tissue size and number is important for lipid storage and small more numerous adipocytes have higher fatty acid turnover and are associated with insulin resistance[80]. Supporting the importance of lipid regulation is the presence of insulin resistance as a core feature of many lipdystrophies[58]. Location of fat, visceral vs. subcutaneous, has also been shown to significantly increase the risk of diabetes[81].

Classically, lipids were thought be the major contributor to insulin resistance through their ability to regulate insulin signaling[58]. Lipid infusions are capable of rapidly inducing insulin resistance and impairing insulin signaling, observed by decreased IRS and Akt phosphorylation. Intracellular lipid accumulation is comprised of triacylglycerols, diacylglycerol (DAG)s, fatty acyl-CoAs and ceramides. Elevations of DAGs, fatty acyl-CoAs, and ceramides in insulin-sensitive cells inhibits insulin signaling by activating the allosteric regulators aPKCθ and aPKCε, JNK, and IKKβ[10, 58, 80]. However, intracellular lipid accumulation does not always induce insulin resistance. For example, in well-trained insulin-sensitive athletes, there is a buildup of intracellular lipids

in their skeletal muscle. This disconnect between lipid excess and insulin resistance is referred to as the athlete's paradox[10, 58]. Isocaloric high-fat diets, moreover, do not cause insulin resistance, but rather, improve lipid metabolism and insulin sensitivity, and decrease fasting insulin, hepatic glucose output, and glucagon[82]. The regulation of signaling by fatty acids is thus not a clear-cut cause of insulin resistance. New consideration of available studies investigating intracellular lipid accumulation has introduced the prospect that impaired insulin signaling might be a consequence rather than a cause of insulin resistance[11].

Chronic inflammation is consistently shown to be a risk factor for insulin resistance and has been demonstrated experimentally to induce insulin resistance in humans and mice. Chronic inflammation manifests through macrophage infiltration in adipose tissue and in the liver—it is unclear if macrophages infiltrate skeletal muscle in a deleterious way[10, 63, 80, 83]. Inflammation is activated by macrophage release of cytokines TNFα, IL1β, IL-6, and C-reactive protein. Cytokines activate allosteric regulators JNK and IKKβ and the pseudosubstrates in the SOCs family[10, 63, 80]. Cytokines also reduce the gene expression of insulin signaling genes and induce a vicious cycle through activation of NF-κβ by IKKβ, inducing the transcription of more cytokines[10, 80]. Inhibition of cytokine activity and reversal of inflammation improves insulin sensitivity in diet-induced insulin-resistant mice and is an important causal mechanism of insulin resistance[80, 84].

Mitochondrial dysfunction and ER stress are less clear mediators of insulin resistance. There is evidence that these two mediators of insulin resistance are initiated by insulin resistance, and afterward propagate the further deterioration of insulin sensitivity.

Mitochondrial dysfunction can affect insulin sensitivity in two pathways. Dysfunction is mediated by either fewer mitochondria or inefficient β-oxidation of fatty acids resulting in impaired ATP generation. The result is an accumulation of fatty acid acyl-CoAs and DAGs[80]. The other possible mechanism is the overproduction of reactive oxygen species (ROS) by mitochondria and peroxisomes[80, 85]. Excessive ROS production allosterically inhibits insulin signaling by activating serine/threonine kinases important for regulating proximal signaling[80].

Some evidence suggests increased intracellular lipids produce ER sheer stress and disrupt cellular homeostasis, leading to protein misfolding and protein unfolding. ER stress activates an unfolded-protein response that ultimately activates JNK and IKKβ. Overexpression of chaperone proteins protects against diet-induced insulin resistance and knockout of chaperones facilitates insulin resistance[80]. These mechanisms appear to be most important in the liver, there is evidence for ER stress as a mechanism in adipose tissue and skeletal muscle but the importance of ER stress in these tissues remains unclear[86].

Hyperinsulinemia, hyperglycemia and hyperleptinemia, can independently induce insulin resistance in cells and humans[27, 29, 76, 87, 88]. Signaling intermediates are allosterically inhibited, and the insulin receptor endocytosed, in response to hyperinsulinemia. Hyperglycemia can inhibit insulin receptor affinity for insulin and affect transcriptional regulation. Hyperleptinemia inhibits IRS by transmitting a signal to a SOCs protein. Hyperinsulinemia, hyperglycemia and hyperlipidemia alone or in combination also increase glucose flux through the HBP increasing inhibitory O-linked glycosylation of signaling molecules[63]. Importantly, the increased flux through HBP will

also result in glycosylation of transcription factors important for mediating cholesterol accumulation in the plasma membrane[78].

1.D.4. Temporal pattern of diet-induced insulin resistance

Animal studies examining the effects of high-fat diets on metabolism have not determined the mechanisms responsible for early, diet-induced insulin resistance[29]. Discovering how skeletal muscle insulin sensitivity is impaired early in the setting of high-fat-feeding is critical for our understanding of glucose intolerance and skeletal muscle insulin resistance, an early manifestation of type 2 diabetes development[7, 13, 24, 89]. It is appreciated that animals fed a high-fat diet undergo a series of metabolic and physiologic changes occurring as early as 24-hours after diet initiation[90-93]. For example, two days following a high-fat feeding challenge, skeletal muscle glucose uptake remains normal despite decreased glycolysis, increased glycogen synthesis, and increased intracellular glucose-6-phosphate[94].

Three days of high-fat-feeding cause mice and rats to present with glucose intolerance and insulin resistance because of defects in skeletal muscle and hepatic insulin action[90, 91]. Around this time blood glucose levels plateau through the influence of compensatory hyperinsulinemia[95]. Total GLUT4 protein content in skeletal muscle remains unchanged in the first 3-4 weeks[96]. In contrast, however, insulin-stimulated glucose transport of animals eating a high-fat diet for 3 weeks is nearly maximally impaired in skeletal and cardiac muscle, and adipose tissue[91].

Perhaps somewhat confounding our understanding are studies that use animals fed a high-fat diet for longer durations (8 to 16 weeks). It is known that insulin resistance precedes inflammation's emergence in muscle, adipose, and hepatic tissue at 8 weeks[84].

Glucose intolerance at 8 weeks can be partly resolved by reversing chronic inflammation, but prior to this there remains a period, between 3 days and 8 weeks, with no clear mechanism of skeletal muscle insulin resistance[29, 84].

Lipid deposition, mitochondrial dysfunction, and ER stress are present in adipose tissue and the liver at 1 week, 4 weeks, and 6 weeks in diet-induced insulin resistance, but these abnormalities are not established in skeletal muscle until 3-4 weeks into high-fat feeding[85, 97]. Initial experiments conducted to investigate the mechanism for early (≤8 weeks) diet-induced insulin resistance discovered insulin receptor binding and signaling defects[92]. However, follow-up studies in this area revealed these mechanisms are likely insufficient in explaining insulin resistance[60, 92]. For example, Grundleger et al. demonstrated a 35% decrease in insulin receptor binding during short-term high fat feeding, but subsequent glucose uptake studies determined only 10-33% of insulin receptor stimulation was necessary for maximal insulin stimulated glucose uptake[10, 92]. These studies demonstrate an excess of insulin receptors and defective insulin binding are not solely responsible for the glucose intolerance in diet-induced insulin-resistant mice[11, 92].

Knockout studies of IRS1 demonstrate its necessity for insulin-stimulated GLUT4 translocation, but impaired GLUT4 translocation from high-fat feeding is still not resolved when IRS1 is bypassed with platelet-derived growth factor receptor stimulation[10, 11]. Knock-in mutations of IRS1 rendering IRS1 resistant to inhibitory phosphorylation is unsuccessful in preventing diet-induced insulin resistance[11]. PI3K activity is reduced by 35% in the skeletal muscle after 3-weeks of high-fat feeding in rats[93]. This PI3K inhibition was not fully explanatory of diet-induced insulin resistance

however, after it was discovered that the administration of okadaic acid, a molecule that bypasses PI3K (also Rac1 and Akt2), to skeletal muscle ex vivo did not correct the insulin resistance[63, 65, 93]. This suggested a distal defective mechanism, perhaps located at the juncture of GLUT4 vesicle translocation and cytoskeletal stabilization. Despite reduced PI3K signaling, diet-induced insulin-resistant animals with resultant decreased Akt phosphorylation, unexpectedly do not display defective Akt-mediated AS160 phosphorylation, relative to their control counterparts[11].

Studies investigating insulin-regulated GLUT4 translocation using inhibitory microRNAs in a dose-response manner found only 5-10% of the total Akt phosphorylation is necessary for insulin to elicit a full GLUT4-mediated glucose transport response in cultured L6 skeletal muscle myotubes[60]. Knockout studies of Akt or Rac1 alone are not sufficient to inhibit GLUT4 translocation. Only a homozygous double-knockout of both genes completely inhibits GLUT4 translocation[46]. It is important to note here that maximum diet-induced skeletal muscle insulin resistance (~50% of control) is associated with a near total (~92%) reduction in insulin stimulated GLUT4 translocation. This association suggests the mechanism for skeletal muscle insulin resistance should almost completely abolish insulin stimulated glucose uptake. The studies discussed in the previous paragraphs, therefore, provide compelling evidence that GLUT4 translocation is not solely dependent on Akt2 activation or actin filament rearrangements.

Interestingly, diminished skeletal muscle glycolysis is seen very early in animals after high-fat feeding, yet does not appear to cause insulin resistance[94]. In fact, glucose uptake remains intact in skeletal muscle after 1 day of high-fat feeding despite a decrease

in glycolysis[94]. These findings are consistent with isotope research and other studies identifying GLUT4 translocation as the rate-limiting step in skeletal muscle glucose disposal[10, 57, 59]. The early discordant findings of decreased glycolysis and stable glucose uptake suggests glucose shunting may be occurring in skeletal muscle just 2 days after the initiation of a high-fat diet. This finding is compelling since glucose shunting through the HBP is a known cause of insulin resistance, and thus, could mediate the subsequent insulin resistance observed after 3 days of high-fat feeding[94].

1.D.5. Hexosamine biosynthesis pathway and insulin resistance

Increased HBP activity has been identified to cause insulin resistance in cell systems, animal models, and human subjects[98]. Excess glucose shunted into this pathway supplies substrate for glutamine:fructose-6-phosphate amidotransferase (GFAT) converting glutamine and fructose-6-phosphate, or experimental provision of glucosamine, into glucosamine-6-phosphate (GlcN-6-P)[98]. This metabolite is then converted into UDP-N-acetylglucosamine (GlcNAc) and then transferred to serine/threonine residues on proteins by the enzyme O-GlcNAc Transferase (OGT)[98]. Targets of OGT include signaling proteins and transcription factors, the functionality of which is modified by glycosylation[99].

In vitro studies in our lab have demonstrated that increased HBP activity causes cellular insulin resistance by increasing plasma membrane cholesterol. Molecular dissection of this revealed increased HBP activity, increased O-GlcNAc-modified levels of the transcription factor specificity protein 1 (Sp1)[78]. Hyperinsulinemia at physiologic levels (250-500pM) increased HBP-mediated O-GlcNAcylation of Sp1, which increased the affinity of Sp1 for the promoter region of HMGR[78]. Inhibiting the rate-limiting

enzyme in the HBP (GFAT) or specifically blocking Sp1 binding to DNA with mithramycin inhibited the hyperinsulinemic-induced cholesterol accumulation and prevented insulin resistance[78]. In vivo investigation by us and others has furthered these findings with evidence that insulin resistance in mice fed a Western-style high-fat diet can be reversed or improved by normalization of excess plasma membrane cholesterol[100, 101]. However, it is not known whether high fat-feeding increases plasma membrane cholesterol through HBP-mediated upregulation of HMGR and if this is independent of inflammation.

Transgenic mice overexpressing GFAT or OGT in skeletal muscle and adipose tissue have peripheral insulin resistance that is mediated by decreased insulin-stimulated GLUT4 translocation[102-104]. GFAT overexpression results in negligible (8.5%) insulin-stimulated GLUT4 translocation, whereas control animals display a significantly greater insulin-stimulated response[102]. Strikingly similar is the observed lack of insulin-stimulated GLUT4 content in skeletal muscle transverse tubule membrane fraction of rats fed a high-fat diet for 4 weeks[105]. Feeding GFAT animals a high-fat diet has no impact on glucose disposal suggesting the cause of skeletal muscle high-fat induced insulin resistance is mediated fully through HBP activation[106].

In humans, skeletal muscle membrane (SMM) cholesterol content is inversely correlated to insulin sensitivity[77]. Human skeletal muscle demonstrates HBP activity is elevated in T2D diabetic subjects and palmitate-induced insulin resistance increases UDP-GlcNAc concentrations in human myotubes[107, 108]. In accord with HBP flux modifying gene expression, recent studies in gene networks have found obesity alters cholesterol metabolism genes predisposing subjects to T2D[109-111]. The identified genes

are related to cholesterol uptake, synthesis, and efflux-producing a molecular profile expected to increase intracellular cholesterol[110]. Epigenetic studies have supported this research with evidence that obesity-driven epigenetic regulation of cholesterol genes increases the risk for T2D[112].

1.E. Cholesterol genes, insulin resistance, and type 2 diabetes

This section is adapted from a review I published in the 2017[113]. The importance of cholesterol genes in insulin resistance was reviewed extensively in this review and the writing of the review served as an important guide for our future studies.

Several human genetic studies suggest a relationship between increased cellular cholesterol levels and alterations in glycemia. Ding et al., quantified the transcriptome and epigenome in monocytes from 1,264 participants in the Multi-Ethnic Study of Atherosclerosis, and found that alterations in a network of coexpressed cholesterol metabolism genes were associated with T2D[114]. This network included 11 genes related to sterol influx (↑*LDLR*, ↓*MYLIP*), synthesis (↑*SCD*, *FADS1*, *HMGCS1*, *FDFT1*, *SQLE*, *CYP51A1*, *SC4MOL*), and efflux (↓*ABCA1*, *ABCG1*), producing a molecular profile expected to increase intracellular cholesterol. Recent examination of multi-tissue transcriptomes and epigenomes suggest that these cholesterol metabolism genes are similarly altered in human adipose tissue[115-120]. Moreover, obesity-driven modifications in the epigenome predicted T2D, independent of conventional risk factors such as BMI and glycemia[116]. Many of the methylation sites responsive to obesity were involved in lipid and lipoprotein metabolism. Identified in this analysis, and many others, was a strong relationship between the methylation of ABCG1 and T2D[116]. As expanded on

below, genetic mutations resulting in decreased circulatory levels of both low-density and high-density lipoproteins are significantly associated with T2D[111].

1.E.1. LDL metabolism

Low-density lipoprotein (LDL) receptor (LDLR)s mediate the cellular uptake of LDL-cholesterol (LDL-C) from the circulation. Myosin regulatory light chain-interacting protein (MYLIP) promotes LDLR degradation. Thus, increased *LDLR* gene expression and/or decreased *MYLIP* gene expression would favor diabetogenic LDLR-mediated cholesterol delivery to skeletal muscle fibers and adipocytes. Consistent with this removal of LDL-C from the blood, lower circulating LDL-C levels have recently been found to be significantly associated with T2D susceptibility[111]. Interestingly, unlike ubiquitous MYLIP tissue expression, proprotein convertase subtilisin/kexin type 9 (PCSK9), which also promotes LDLR degradation, is produced predominantly in the liver. Therefore, PCSK9 inhibitors, unlike the genetic loss of MYLIP, would not be expected to increase cholesterol levels in non-hepatic cells. Whether PCSK9 inhibitors, however, increase T2D risk is not yet fully known[121]. Contrariwise to increased LDLRs and decreased LDL-C associating with T2D, loss-of-function mutations in the LDLR, as seen in familial hypercholesterolemia, protects individuals from T2D risk[122]. In fact, the odds of developing T2D decreased linearly as the severity of familial hypercholesterolemia increased[122], or, from another prospective, as cellular ability to uptake cholesterol decreased.

1.E.2. HDL metabolism

A significant association between genetically determined lower HDL-C and T2D has also been found[111]. Unlike the LDLs that deliver cholesterol to cells, HDLs remove

cholesterol from cells. HDLs originate from the liver, intestine, chylomicron (CM), and very-low-density lipoprotein (VLDL). The liver secretes lipid poor ApoA1 called nascent or precursor HDL. The intestine directly synthesizes these particles. Finally, lipoprotein lipase (LPL)-mediated lipolysis of CMs and VLDLs releases surface ApoA1 and phospholipids that also generate nascent HDLs. This is facilitated by phospholipid transfer protein (PLTP). These liver-, intestine-, CM-, and VLDL-derived nascent HDLs (designated pre-β_1, pre-β_2, and pre-β_3) accept free cholesterol from cell membranes with excess cholesterol. This transfer of free cholesterol to HDLs is mediated by ABCA1, ABCG1, the class B, type 1 scavenger receptor (SR-B1), as well as other cell surface proteins. Following free cholesterol transfer to the surface of the nascent HDLs, it is esterified by lecithin:cholesterol acyltransferase (LCAT) and the formed cholesterol esters move away from the surface to a cholesterol ester-rich core forming a small, spherical, mature HDL particle (designated HDL_3). Through this same LCAT-mediated process HDL_3 accepts cellular free cholesterol, grows, and matures to a form designated as HDL_2. Cholesterol ester transfer protein (CETP) facilitates the transfer of cholesterol esters from HDL_2 to the lower density lipoproteins (VLDL, IDL, LDL) that transit to the liver for excretion. As the HDL_2 particles becomes devoid of cholesterol esters, hepatic lipase hydrolyzes triglycerides and phospholipids that the HDL_2 molecule accumulated and this reconverts HDL_2 to HDL_3. The regenerated HDL_3 cycles back through this pathway of accepting free cholesterol and transitioning to HDL_2 and then back to HDL_3.

Genetic mutations in many of these system components of HDL metabolism tend to increase a carrier's risk for T2D. Perhaps these mutations negatively impact the efficiency of mitigating diabetogenic cholesterol accumulation in skeletal muscle fibers

and adipocytes. For example, Lara-Riegos et al. found T2D susceptibility in Mexican Mestizos was associated with a loss-of-function mutation in ABCA1[123]; however, genetic variation in ABCA1 was not found to predict T2D in other populations[124]. Interestingly, mutation in genes for ApoA1, CETP, SR-B1, and Niemann-Pick disease, type C1 (NPC1) tend to increase a carrier's risk for T2D[125-129]. Loss-of-function mutations in ApoA1, CETP, and SR-B1 would negatively impact HDL functionality in accepting free cholesterol from cells with excess cholesterol. Similarly, a loss-of-function mutation in NPC1, a gene mutated in Niemann-Pick disease that disrupts intracellular cholesterol transport and accumulation in late endosomes and lysosomes, indirectly impedes ABCA1-mediated cholesterol efflux by sequestering this cholesterol transporter in the endosomal compartment[130]. Interestingly, we have found that HBP-mediated increases in cholesterol biosynthesis also results in endosomal membrane cholesterol accumulation and sequesters ABCA1 in that compartment away from the cell surface where it functions to transfer free cholesterol to ApoA1[131]. Mutations in chromosome 9q31 in people with Tangier disease lead to defective ABCA1 transporters and these patients have been reported to have impairments in insulin action and insulin secretion[132]. Peptides have been developed with ABCA1 agonist properties acting like ApoA1 by promoting cellular cholesterol efflux[133]. These peptides were studied in cell lines, diet-induced insulin-resistant mice with or mice without the APOE knockout and had a remarkable anti-diabetic effect. Peptide administration improved insulin-stimulated glucose uptake, insulin secretion and restored glucose and insulin tolerance to levels equal to what is seen in chow fed mice[133].

It has been widely published that statin medications cause insulin resistance and increase the risk of type 2 diabetes. Cellular changes from HMGR inhibition appears to be mediated by increased transcription of SREBPs and two associated microRNAs (miR), miR-33a and miR-33b[134, 135]**.** In response to statins, SREBPs and miR-33a/b function as modulators of cellular cholesterol levels by increasing HMGR and LDLR, and decreasing ABCA1, ABCG1, NPC1, and AMPK, respectively[134, 135]. These metabolic changes are advantageous for reducing circulating blood cholesterol, although an exaggerated compensatory response in adipocytes, pancreatic β-cells, or skeletal muscle fibers could have deleterious consequences on glucose regulation. Circulating levels of these two miRNAs are associated with insulin resistance and type 2 diabetes in the elderly[136].

This cholesterol network is of interest for our understanding of membrane cholesterol accumulation. Unlike cells, *in vivo* cholesterol handling is dynamic with many pathways available to cells in need of shedding excess cholesterol. Insulin resistance in mice with skeletal muscle GLUT1 overexpression can only be identified *ex vivo*, whereas mice overexpressing GFAT do not have *ex vivo* measured insulin resistance[137-139]. Furthermore, GFAT overexpression in adipocytes is sufficient to cause skeletal muscle insulin resistance, but it has been reported that GFAT overexpression solely in skeletal muscle does not impair insulin-stimulated glucose uptake[137, 140]. The discrepancy between these studies may be a result of cholesterol transport. The effect of HBP on skeletal muscle cholesterol accumulation may be blunted in the absence of any adipose tissue pathology. The studies identifying GLUT1-mediated resistance only i*n vitro* also suggest a protective effect from metabolically healthy fat. Teasing out the patterns of cholesterol genes in the fat and muscle of high-fat fed mice may start us on

the path of understanding the complex interplay between muscle and fat. Comparison of these tissues between high fat and GFAT transgenic mice may also help us determine which cholesterol genes are regulated by HBP and which genes if any are influenced by another mechanism.

1.F. Hypothesis and aims

While insulin resistance is admittedly associated with multiple cellular defects, evidence suggests muscle cholesterol accumulation may be an early etiological factor. The studies presented show a sequence in disease pathophysiology beginning with impaired glycolytic flux and an apparent shift of glucose to alternative pathways[94]. Shortly after this shift an undefined mechanism initiates skeletal muscle insulin resistance[29, 94]. I predict skeletal muscle cholesterol accumulation is a reversible mitigating factor in early skeletal muscle insulin resistance. Moreover, I expect high fat feeding increases glucose flux into the HBP thereby promoting HMGR expression by increasing the cellular level of O-GlcNac-Sp1.

Studies in my dissertation will determine 1) Whether skeletal muscle cholesterol accumulation occurs early in the setting of high-fat feeding and if this accumulation impairs GLUT4 translocation; and 2) If high-fat feeding cholesterol accumulation occurs because of increased HBP regulation of transcription factors and the cholesterol genes they regulate[78].

Chapter 2. Materials and methods

2.A. Mice

Male C57BL/6NJ (6N) mice were obtained at 6 weeks of age from Jackson Laboratory, Bar Harbor, ME. All mice were singly housed in conventional cages and maintained on a 12-h light/dark cycle. Body mass was recorded daily or weekly. The IUSM Institutional Animal Care and Use Committee approved all animal protocols.

2.B. Mouse selection

C57BL/6J (6J) mice are one of the most commonly used mouse model for high fat-diet experiments investigating insulin resistance and T2D research. These animals were selected for their ability to become obese and glucose intolerant under conditions of high fat feeding. One limitation with this model is the absence of the Nicotinamide Nucleotide Transhydrogenase (NNT)[90]. This gene is responsible for reducing NADPH in mitochondria. The absence of this gene leads to a lower concentration of the reduced form of glutathione and subsequent increase in ROS levels. A major effect of this mutation is a reduction in insulin secretion in response to a glucose load. The physiology of C57BL/6J mice is, in this manner, unlike humans. While humans have a biphasic response to glucose during an intravenous glucose tolerance test (IVGTT), 6J mice display no detectable insulin response to an IVGTT[141]. In response to an arginine bolus, 6J mice insulin secretion is negligible, where as in humans a bolus of arginine would result in a robust insulin secretion response[141].

In comparison to 6J mice, the 6N mouse model has the NNT gene[90]. When subjected to an IVGTT 6N mice demonstrate a biphasic insulin response and a bolus of arginine will elicit a robust insulin secretory response[141]. 6N mice like 6J mice gain

weight and develop glucose intolerance when fed a high fat diet. The 6N mice, however, develop hyperinsulinemia 1 week after glucose intolerance is achieved. Hyperinsulinemia is a characteristic pathophysiologic feature of human insulin resistance seen up to 10 years prior to the onset of T2D, is indistinguishable from the development of insulin resistance, and is a mediator of worsening insulin resistance[27, 76, 78, 141]. For these reasons, we consider the 6N mice to be a more representative model for preclinical study of insulin resistance.

2.C. Diet

Upon arrival to our facility, all mice had free access to water and standard laboratory chow for 1 week. Following this 1-week acclimation period, all mice received a low-fat (3.85 kCal/gram) diet containing 20% kcal from protein, 70% kcal from carbohydrates, and 10% kcal from fat (D01030107, Research Diets Inc., New Brunswick, NJ) for 1 week to adapt to the modified diet. This low fat, as well as the high-fat (4.73 kCal/gram), diet represented modified forms of the standard low fat (D12450B) and high fat (D12451) diets from Research Diets Inc., with adaptations regarding type of fat (palm oil instead of lard) and carbohydrates, to better mimic the fatty acid (FA)/carbohydrate composition of the average human diet in Western societies [100, 142]. Following this 2-week acclimation period, mice were either left on the low-fat diet or switched to the high fat diet containing 20% kcal from protein, 35% kcal from carbohydrates, and 45% kcal from fat (D01030108) for 1 week. This high fat diet mimics the percent of saturated to monounsaturated to polyunsaturated FAs (40:40:20). A subgroup of the 1-week high-fat-fed animals were switched back to the low-fat diet for 1 week following the high fat diet challenge and a subgroup of low-fat-fed animals were low fat fed for 2 weeks.

2.D. Duration of diet

6N mice will be acclimated to a palmitate based low fat diet (D01030107, Research Diets Inc.) for 1 week consisting of 70% kcal from carbohydrates, 10% kcal from fat, and 20% kcal from protein. After the acclimation period half of the mice will be maintained on the low-fat diet while the other group will be randomly selected and switched for 1 week to a high fat diet (D01030108) consisting of 35% kcal from carbohydrates, 45% kcal from fat, and 20% kcal from protein. All mice will acclimate to palmitate with low fat feeding for 1 week and then diet challenged.

There is a considerable amount of disagreement in the literature concerning the predominance of hepatic vs. skeletal muscle insulin resistance after 1 week of feeding animals a high fat diet. These differences appear to be related to the methodology used for detecting insulin resistance[94, 143, 144]. Using the hyperinsulinemic-euglycemic clamp, the gold standard method for investigating insulin resistance, Kim et al. demonstrated that the identification of either hepatic or skeletal muscle insulin resistance depends on the dose of insulin used in the study[94]. Briefly, their study showed that rats subjected to a high fat diet for 1 week have hepatic insulin resistance when tested with submaximal (30 $pmol \cdot kg^{-1} \cdot min^{-1}$ or 4.32 $mU \cdot kg^{-1} \cdot min^{-1}$) insulin during the hyperinsulinemic-euglycemic clamp, whereas maximal (300 $pmol \cdot kg^{-1} \cdot min^{-1}$ or 43.2 $mU \cdot kg^{-1} \cdot min^{-1}$) insulin stimulation identifies peripheral insulin resistance[94]. Clamp studies indicating a only hepatic insulin resistance at 1 week use insulin doses ≤ 4 $mU \cdot kg^{-1} \cdot min^{-1}$, whereas studies identifying muscle insulin resistance use insulin doses $\geq 8 mU \cdot kg^{-1} \cdot min^{-1}$ [84, 94]. The differences in these results is due to the dose required to achieve maximal insulin stimulated glucose uptake and maximum inhibition of endogenous glucose production[143]. Dose response curves in

mice have demonstrated >10 $mU \cdot kg^{-1} \cdot min^{-1}$ is needed to achieve maximal stimulation of skeletal muscle glucose uptake and maximal inhibition of endogenous glucose production[143, 145].

Ex-vivo studies investigating skeletal muscle insulin resistance at either 10 days or 7 days have found skeletal muscle resistance and no resistance, respectively. The ex vivo studies differ in four important ways, the respective use of animal model rat vs. mouse, the insulin concentration physiologic vs. supraphysiologic, the amount of time the animal was exposed to the diet, and most importantly the muscle fiber type; e.g., slow oxidative (e.g., soleus) vs. fast glycolytic (e.g., extensor digitorum longus). Slow oxidative muscle has the highest capacity for glycogen storage, has a vastly greater capacity for glucose uptake, has the greatest concentration of GLUT4, and is most sensitive to diet-induced changes in insulin sensitivity making soleus selection in ex vivo experimentation an important consideration to identify subtle changes in skeletal muscle insulin sensitivity[90, 92, 105, 146]. These studies agree with the skeletal muscle insulin resistance indicated by decreased pAKT levels and pGSK observed by Kelsey H. Fisher-Wellman and colleagues comparing the role of high fat feeding on 6N and 6J mouse metabolism[90].

2.E. GFAT animals

In collaboration with Dr. Donald McClain we recently rederived his transgenic mouse model that overexpresses fat/muscle GFAT and display defective insulin-stimulated GLUT4 regulation and glucose disposal[102, 103]. In these mice, transgene expression in fat/muscle is targeted with the GLUT4 promoter. These transgenic mice overexpress GFAT at approximately two-fold higher levels. Considering that the HBP

accounts for ~2% of total cellular glucose flux[147], increasing flux to ~4%, will still not reach a level that will affect glucose availability for oxidative or nonoxidative metabolism. Hebert et al. found that this increased flux through the HBP pathway led to weight-dependent hyperinsulinemia in random-fed mice and using the hyperinsulinemic–euglycemic clamp technique confirmed that GFAT transgenic mice develop insulin resistance[103]. Of importance, a measured decrease in glucose uptake by GFAT Tg muscle was associated with a disruption in insulin-regulated GLUT4 translocation[102], consistent with our model.

2.F. Cholesterol shuttling experiments

Methyl-β-cyclodextrin from (Sigma molecular weight = 1320) was made into a stock concentration of 5mM by dissolving 6.6 mg methyl-β-cyclodextrin /ml saline. Cholesterol removal studies were performed using a subcutaneous dose of 50mg/kg which has been found to be tolerable and efficacious[101]. High fat fed mice were injected with 5mM methyl-β-cyclodextrin twice a week once at 3 days following the administration of the high-fat diet and again at 6 days of high fat feeding. Mice physiologic studies were performed at day 7 of high fat feeding.

Cholesterol addition was performed using 5 mM methyl-β-cyclodextrin laden with cholesterol at a 4:1 molar ratio of methyl-β-cyclodextrin:cholesterol. Mice were injected twice in the morning prior to animal testing. Two 50 mg/kg subcutaneous doses were administered an hour apart, once at the time of fasting and once an hour later. The 4:1 molar ration has been shown to shuttle cholesterol into cellular membranes *in vivo*[148]. Experimental procedures were performed after the usual 6 hour fasting period.

Cholesterol laden methyl-β-cyclodextrin (4:1 molar ratio) was made as follows: Cholesterol from Avanti Polar Lipids (molecular weight = 386.66) was dissolved 5mg/ml in 20 ml of cholorform:methanol 1:1 mixture or 0.25mM cholesterol in 10 ml solution was made as stock cholesterol concentration. 1.933 ml of stock cholesterol was evaporated in a glass tube under a gentle stream of nitrogen gas so that the cholesterol film was all that remained in the tube. 10ml of 5mM methyl-β-cyclodextrin was added to the glass tube containing the cholesterol film. The 10ml methyl-β-cyclodextrin:cholesterol solution was sonicated in a bath sonicator for 3-5 minutes at 37 degrees Celsius at 65 kHz. The solution was incubated in a water bath at 37 degrees Celsius for 48 hours at 75 rounds per minute. Methyl-β-cyclodextrin:cholesterol solution was filtered through a 0.45μM syringe prior to subcutaneous injection. All cellular and physiologic testing was performed on subcutaneous injected saline controls.

2.G. Sp1 inhibition

Mice fed a high fat diet for one week were injected daily intraperitonially with a dose of 150 μg/kg mithramycin (Cayman Chemical, Ann Arbor, MI, USA). This dose has been shown to be tolerable and efficacious in a study of Alzheimer's using APPswe/PS1dE9 mice[149]. Mice physiologic studies were performed at day 7 of high fat feeding. Mithramycin 1g tubes were solubilized in 2 ml of saline. This mithramycin solution was filtered through a 0.45μM syringe prior to intraperitoneal injection. All cellular and physiologic testing was performed on treated mice and controls intraperitoneally injected with saline.

2.H. Glucose, insulin, and pyruvate tolerance tests

For the intraperitoneal glucose, insulin, and pyruvate tolerance tests, mice that were fasted for 5-6 hours were administered glucose (2 g/kg mass i.p.), insulin (0.5 U/kg mass i.p.), or pyruvate (2 g/kg mass i.p.), respectively. Tail vein blood glucose was measured at times indicated with an AlphaTRAK blood glucose meter (Abbott Laboratories, Inc. Alameda, CA). Glucose-stimulated insulin secretion was also performed to identify the presence of hyperinsulinemia. At the 0- and 15-minute time points following the glucose injection for the glucose tolerance test, plasma was collected for insulin measurements. The 15-minute time point was selected to allow for data comparison with previous research [90]. Insulin tolerance test glucose was analyzed for the first 30 minutes and pyruvate tolerance glucose was collected over a period of 90 minutes.

2.I. Liver analyses

To determine hepatic glycogen, approximately 30 mg of mouse liver was digested in 0.5 ml of 1M KOH for at least 30 minutes at 70°C. 100 µl of the liver digest was then neutralized with 17 µl of 17.4 M acetic acid followed by incubation with 500 µl of 0.3 M acetate buffer containing 0.5% amyloglucosidase (Roche) for 2 hours at 37°C. 2 µl was added to 200 µl Trinder Glucose Oxidase reagent (Pointe Scientific) and incubated for 30 minutes at room temperature. For triglyceride, approximately 0.3 g of mouse liver was added to 2 ml of ice-cold HES buffer (in mM: 250 sucrose, 20 HEPES, 2 EGTA and 3 NaN3, pH 7.4) containing freshly added protease inhibitors (in µM: 200 PMSF, 1 leupeptin and 1 pepstatin A) and subsequently homogenized. 10 µl of 20% NP40 was added to 200 µl of homogenate. This mixture was rotated for 10 minutes at 4°C followed

by boiling for 10 minutes. Samples were then centrifuged at 14,000 rpm for 10 minutes at room temperature. 5 µl of supernatant was then added to 200 µl of GPO reagent (Pointe Scientific) and incubated for 15 minutes at room temperature. Absorbances for both were then measured at 505nm.

2.J. Actin analyses

A thin slice of mixed hindlimb skeletal muscle was labeled, mounted in Vectashield, and analyzed via confocal microscopy (LSM 510 NLO; Zeiss, Thornwood, NY) as previously described [71]. Following fixation in 2% paraformaldehyde/PBS for 2 h, tissues were washed with PBS and stored at 4 °C. A small section was excised from each tissue and incubated in 0.2% Triton X-100/0.05% Tween 20/PBS for 30 min at 25 °C. The sections were then rinsed three times in 0.05% Tween 20/PBS and blocked in 2% BSA/0.05% Tween 20/PBS (for actin) for 60 min at 25 °C. Sections were then incubated overnight at 4 °C in mouse IgM anti-human F-actin (Serotech Oxford, UK), antibodies diluted 1:50 in blocking buffer. Samples were then washed extensively in 0.05% Tween 20/PBS. Sections were incubated for 45 min at 25 °C in 1:50 rhodamine-conjugated donkey anti-mouse IgM (for actin labeling), 1:50 fluorescein isothiocyanate-conjugated donkey anti-latrunculin B for 1 h. Muscles were then incubated in the presence or absence of 13.3 nM insulin and fixed in 2% paraformaldehyde/PBS for 2 h. Muscles were then processed and stained for: A cortical actin as described under Following secondary antibody incubation, samples were washed extensively with 0.05% Tween 20/PBS, rinsed with ddH2O, mounted to slides in Vectashield (Vector Laboratories, Inc., Burlingame, CA), and examined via confocal microscopy (Zeiss LSM 510 NLO Confocal Microscope). All images were taken in the same focal plane of the section and

under identical microscopic parameters. Images shown are representative of three to five fields from each sample. Prior to imaging, all samples were de-identified to ensure an objective analysis. All images were taken in the same focal plane of the section and under identical microscopic parameters. Images shown are representative of 5-7 fields from each sample.

2.K. Membrane preparation cholesterol analyses and protein concentrations

Mixed hindlimb skeletal muscle triad enriched membrane was obtained as previously reported [101]. We isolated triads consisting of T-tubule, sarcolemma, and sarcoplasmic reticulum using a procedure that has been shown to be as effective at procuring T-tubule enriched triads as pure as the sucrose density gradient method[150]. Skeletal muscle was homogenized in ice-cold HES buffer with a Polytron PT-10 homogenizer 3 times in 10-second bursts. After homogenization the sample was centrifuged at 1380g for 30 minutes, after which the supernatant was filtered through cheese cloth and the pellet was resuspended and centrifuged again at 1380g for 30 minutes. The supernatant from the second centrifugation was combined with the supernatant from the first spin and centrifuged at 17,000g for 30 minutes. The supernatant was discarded, and the pellet was homogenized in HES buffer and centrifuged at 1380g for 30 minutes. The supernatant was filtered through cheese cloth centrifuged at 17,000g for 30 minutes. The resulting pellet was then resuspended in HES homogenized and centrifuged at 17,000g for 30 minutes. The resulting pellet was then reconstituted in 0.2 ml of HES buffer and stored at -80 degrees Celsius for analysis[101, 150]. Triad cholesterol content was assayed using the Amplex Red Cholesterol Assay Kit

(Molecular Probes) [76]. Protein concentrations for all samples were quantified using the Bio-Rad DC protein assay.

2.L. RNA analyses

Mixed hindlimb muscle from mice were lysed using Qiagen QIAshredder and RNA was isolated using an RNeasy mini kin (Qiagen). Purified RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosytems). Reactions were performed in a 96-well plat using the ABI Prism &00 Sequence Detection System (Applied Biosystems). Each reaction contained the following: 12.5 µl of SYBR GREEN (Applied Biosystems), 200nM of each primer, 3 µl of cDNA, and RNase free water to a total volume of 25 µl. The PCR conditions used were 95°C for 15s, 60°C for 40s. Cycle threshold values were obtained and normalized to 36B4. The ΔΔCT method was used to determine relative expression levels[151].

2.M. GLUT4 analyses

GLUT4 translocation was assessed using mixed hindlimb skeletal muscle. Mixed hindlimb skeletal muscle was rapidly harvested from mice that were fasted for 14-18 hours, 30 minutes following an intraperitoneal glucose (2 g/kg mass i.p.) injection. Skeletal muscle membrane cell surface pellets were obtained as described above (Membrane Preparation) and subjected to Western immunoblot analysis. Samples containing an equal amount of total protein were loaded onto a 10% acrylamide resolving gel. Protein concentration was determined using the DC Protein Assay Kit (Bio-Rad). Protein was transferred from the gel to a nitrocellulose membrane. Equal protein loading per lane was further verified using the Revert Total Protein Stain (Li-Cor) and used to normalize GLUT4 to total protein. Anti-GLUT4 antibodies (Cell Signaling Technology)

were used to detect GLUT4 and quantification of the obtained image was performed to determine Triad GLUT4 per total protein content.

2.N. Insulin signaling analyses

GLUT4 translocation was assessed using mixed hindlimb skeletal muscle. Mixed hindlimb skeletal muscle was rapidly harvested from mice that were fasted for 14-18 hours, 15 minutes following an intraperitoneal glucose (2 g/kg mass i.p.) injection. Skeletal muscle whole cell lysate was homogenized in an NP40, EDTA, hepes buffer with aprotinin, leupeptin, PMSF, pepstatin, sodium fluoride, sodium chloride, and sodium pyrophosphate. Samples containing an equal amount of total protein were loaded onto a 10% acrylamide resolving gel. Protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad). Protein was transferred from the gel to a nitrocellulose membrane. Equal protein loading per lane was further verified using the Revert Total Protein Stain (Li-Cor) and used to normalize pAkt to total protein. Anti-pAkt (Ser 474) antibodies (Cell Signaling Technology) were used to detect pAkt and quantification of the obtained image was performed to determine pAkt per total protein content. Membranes were stripped and re-probed for total Akt. Anti-Akt2 (Cell Signaling Technology) antibody was used to probe for total Akt2.

2.O. Statistical analyses

Values presented are means ±SEM. The significance of differences between means was evaluated by ANOVA. Where a difference was indicated by ANOVA, Sidak's post-hoc test was conducted to compare differences between groups with high fat group only. Statistical comparisons of body mass, areas under or above the curve (AUC and AAC, respectively), liver glycogen and triglycerides content were performed by a

two-tailed Student's *t* test when only two groups were analyzed. Area under or above the curve with respect to the increase was calculated for all GTT, ITT, and PTT measures using newtons method. GraphPad Prism 7 software was used for all analyses. $P<0.05$ was considered significant.

Chapter 3. Results

3.A. Mice fed a high-fat diet for one-week display cholesterol-associated reversible metabolic derangements.

Impaired metabolic responses have been demonstrated in 6N mice within 1 week of high fat feeding[90]. For our studies, 6N mice were fed either a 10% low-fat (LF) or 45% high-fat (HF) diet. We divided mice into multiple cohorts, so each variable measured was performed exactly following 1 week of the diet challenge. Figure 3A shows a slight but significant 1.17 g gain in body mass in mice fed a HF diet compared to the LF-fed mice when comparing all mice (n=41 per group). Using 6 mice per group we performed glucose-, insulin-, and pyruvate tolerance tests. One week of HF feeding significantly impaired glucose tolerance (Figure 3B inset). While this acute HF-feeding challenge did not impair insulin tolerance (Figure 3C, inset), we did observe a significant increase in glucose-stimulated insulin secretion during the glucose tolerance test (Figure 3D, compare bars 3 and 4) at the 15-min time point, consistent with the development of whole-body insulin resistance. In a separate cohort of mice, fasting serum insulin levels trended to be slightly increased following 1-week of HF feeding; yet this did not reach statistical significance (Figure 3D, compare bars 1 and 2). On the other hand, fasting glucose levels measured during the glucose and insulin tolerance test were significantly elevated (see Figures 3B [LF, 160.8±13.2 vs. HF, 211.3±4.03 mg/dL, p=0.0044] and 3C [LF, 154±5.7 vs. HF, 188.6±6.4 mg/dL, p=0.0038]). To assess whether hepatic insulin resistance contributed to this increase in fasting blood glucose we performed a pyruvate tolerance test. Again, mice used for this test also showed a similar increase (LF, 161±4.9 vs. HF, 188.7±7.6 mg/dL, p=0.0122) in fasting blood glucose following 1 week of high-fat feeding, yet these mice did not display pyruvate intolerance (Figure 3E). In addition to

assessing pyruvate-induced hepatic glucose output, we measured glycogen and triglyceride levels in livers from LF- and HF-fed mice, and the levels of each were unaffected by the HF-feeding challenge (see Figures 3F and G) further supporting the lack of a pronounced hepatic insulin resistance in these 1-week HF-fed mice.

In addition to the study of the LF- and HF-fed groups, we wanted to determine the mechanism for the metabolic benefits of HF diet withdrawal. We reasoned that like seen in other animal and human studies, placing mice back on a LF-diet for 1 week following the 1-week HF feeding challenge should mitigate some or all the measured metabolic derangements. For this study we used LF-fed mice that remained on the diet for 2 weeks as a control (LF^2). The mice that were HF-challenged for 1 week and then placed on a LF diet for an additional 1 week (denoted reversed fat, RF) still trended to be heavier (see Figure 4 [LF_2, 23.47±0.34 vs. RF, 24.18±0.41 g, p=0.2004]), yet this difference was not statistically significant. Figures 4B-D show that glucose and insulin tolerance and glucose-stimulated insulin secretion in the RF group were not different from the LF_2 control. While the mice we used for the insulin tolerance test showed an elevation in fasting glucose (LF_2, 161.4±3.84 vs. RF, 177.3±2.4 mg/dL, p=0.0052), this was not the case with the mice used for the glucose tolerance test (LF_2, 175.2±4.7 vs. RF, 180.2±4.8 mg/dL, p=0.4725).

To determine causality of HF mediated insulin resistance and the associated factors attributed to reversal of diet we treated HF-fed mice with the cholesterol-lowering agent methyl-β-cyclodextrin (CD). Body mass and metabolic testing was determined on saline treated LF-fed, saline-treated HF-fed and CD-treated HF-fed.

As shown in Figure 5A, body mass was not different between the groups. Again, with this cohort, 1 wk of HF feeding impaired glucose tolerance (Figure 5B, inset) as well as elevate fasting blood glucose (LF, 143.7±11.4 vs. HF, 194.4±7.7 mg/dL, p=0.0086). In the HF-fed mice treated with CD glucose tolerance and elevated blood glucose levels were completely normalized. In addition, the CD treatment strongly trended (p=0.07) to lower the elevated serum insulin in HF-fed mice 15 min following a glucose injection to a level that was not significantly different from the LF group (Fi. 5C). We also determined caloric intake of each group during their diet challenge and treatments. As shown in Figure 5D, HF-feeding was associated with a higher caloric intake compared to the LF-fed and RF-fed animals (compare bars 1-3). Notably, we observed a slight, yet significant decrease in caloric intake in the RF group compared to the LF group. Importantly, HF-fed mice treated with CD were found to consume the same amount of calories as the HF saline group (Figure 5D, compare bars 2 and 4).

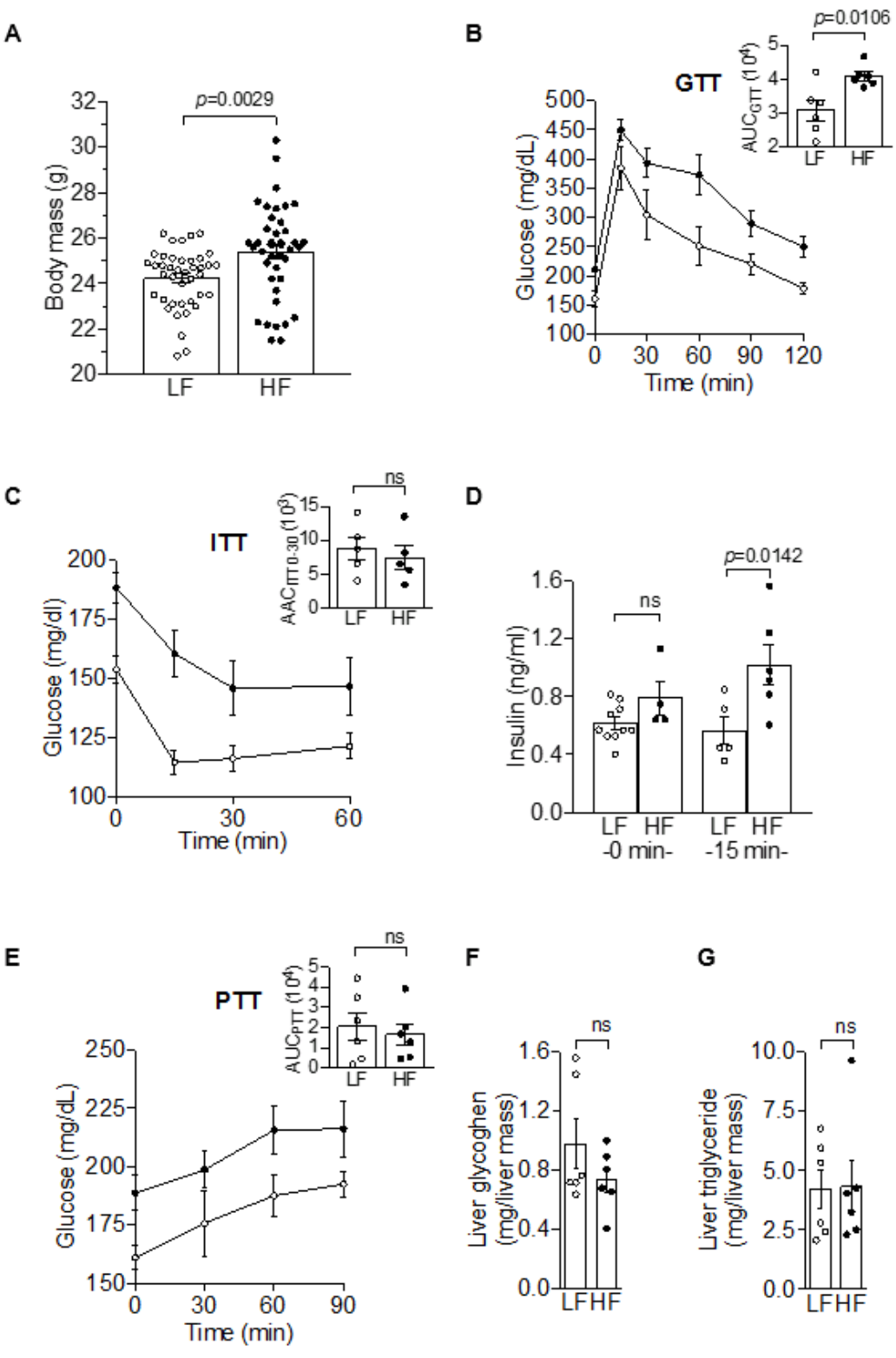

A
p=0.0029
Body mass (g)
32
30
28
26
24
22
20
LF
HF
B
GTT
p=0.0106
AUC_GTT (10^4)
5
4
3
2
LF
HF
Glucose (mg/dL)
500
450
400
350
300
250
200
150
100
0
30
60
90
120
Time (min)
C
ITT
ns
AAC_ITT 0-30 (10^3)
15
10
5
0
LF
HF
Glucose (mg/dl)
200
175
150
125
100
0
30
60
Time (min)
D
p=0.0142
ns
Insulin (ng/ml)
1.6
1.2
0.8
0.4
0.0
LF HF
-0 min-
LF HF
-15 min-
E
PTT
ns
AUC_PTT (10^4)
5
4
3
2
1
0
LF
HF
Glucose (mg/dL)
250
225
200
175
150
0
30
60
90
Time (min)
F
ns
Liver glycoghen (mg/liver mass)
1.6
1.2
0.8
0.4
0.0
LF HF
G
ns
Liver triglyceride (mg/liver mass)
10.0
7.5
5.0
2.5
0.0
LF HF

Figure 3. Glucose intolerance and insulin resistance develop within 1 week following a high-fat feeding challenge. (A) Body mass after 1 week of low-fat (LF) or high-fat (HF) feeding. **(B)** Blood glucose measured after an intraperitoneal injection of glucose (2g/kg) or **(C)** insulin (0.5 U/kg). **(D)** Serum insulin concentrations before (0-min) and 15 minutes following an intraperitoneal injection of glucose (2g/kg). **(E)** Blood glucose measured after an intraperitoneal injection of pyruvate (2g/kg). **(F)** Liver glycogen content. **(G)** Liver triglyceride content. Open and closed circles denote LF-fed and HF-fed animals, respectively. All data are mean ±S.E. for n=6 per group. *P<0.05 vs. LF control group.

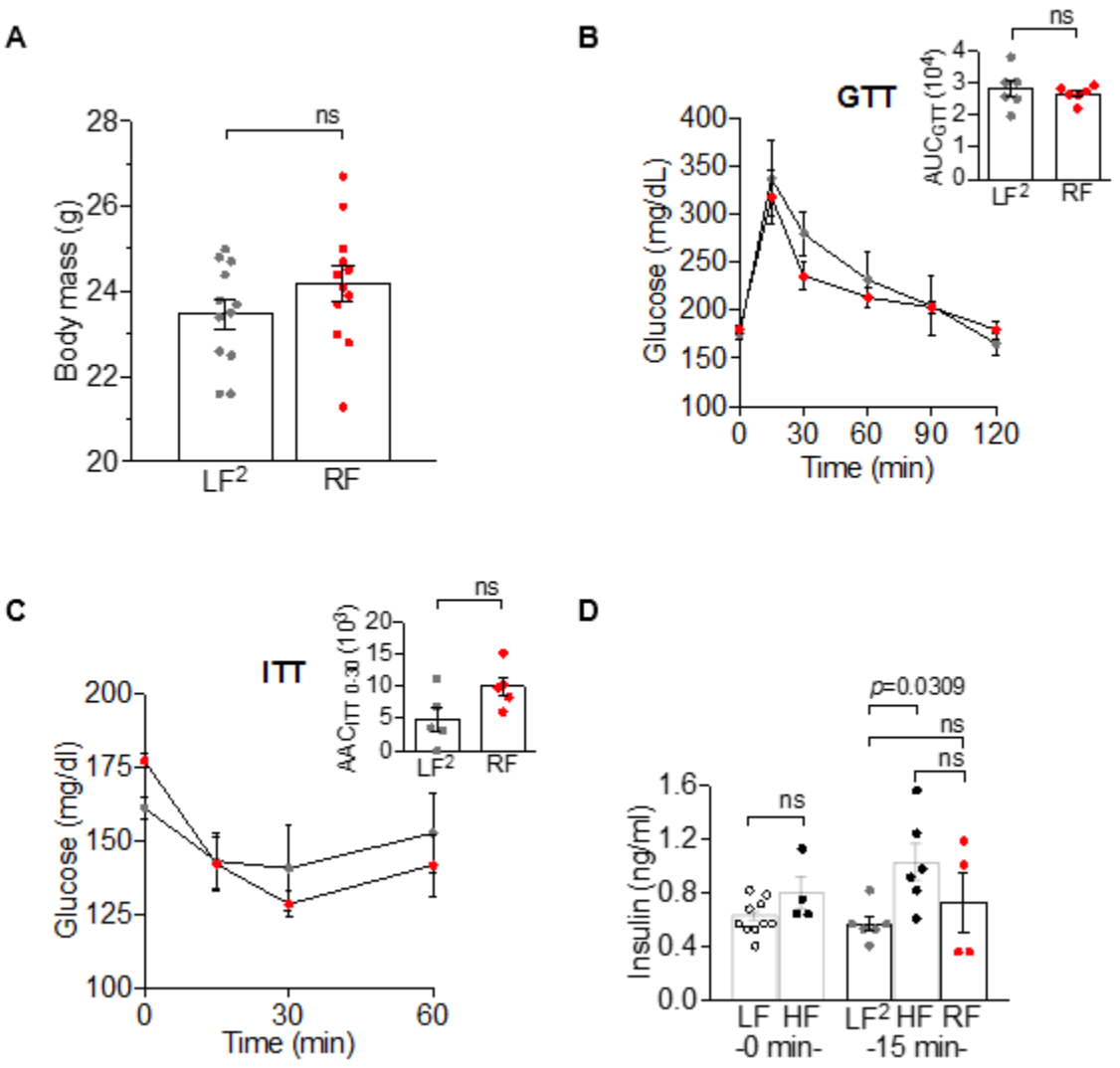


Figure 4. Low-fat feeding mice that were high-fat fed for 1 week mitigates glucose intolerance and insulin resistance. (A) Body mass measured at the end of reversal period. **(B)** Blood glucose measured after an intraperitoneal injection of glucose (2g/kg) or **(C)** insulin (0.5 U/kg). **(D)** Serum insulin concentrations before (0-min) and 15 minutes following an intraperitoneal injection of glucose (2g/kg). Boxes with pale outlines in **(D)** are replicated from Figure 1 and statistics are from an ANOVA comparing all groups. All data are mean ±S.E. Reversal Fat (RF) identifies mice fed a HF diet for 1-

week and switch back to a low-fat for a week. LF^2 denotes low fat controls that were on a low-fat diet for the same duration of time as the RF group.

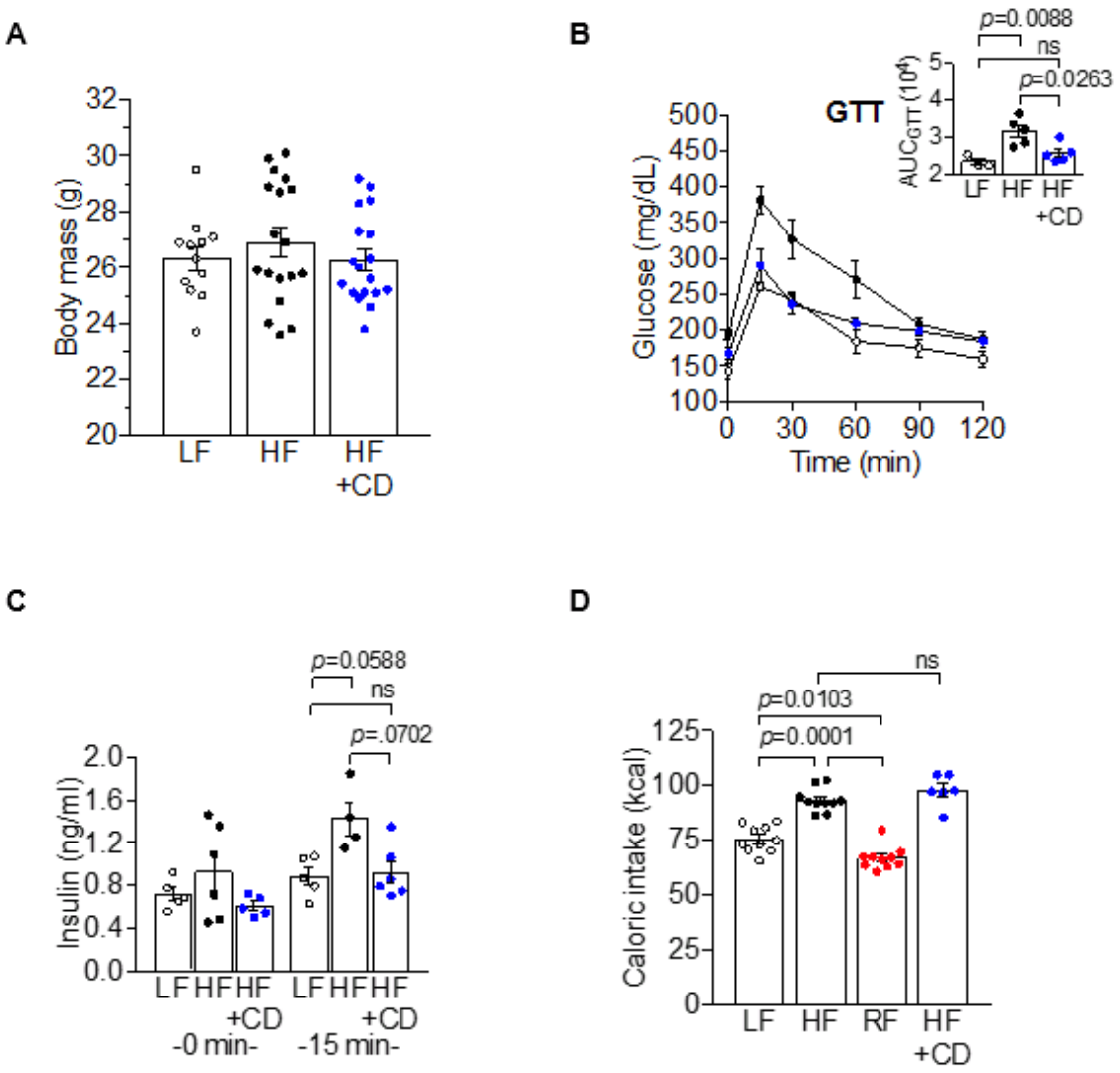


Figure 5. Administration of nascent methyl-β-cyclodextrin to 1-week high-fat-fed mice mitigates glucose intolerance and insulin resistance. (A) Body mass for LF and HF saline controls and HF methyl-β-cyclodextrin (HF +CD) treated animals. **(B)** Blood glucose measured after an intraperitoneal injection of glucose (2g/kg) or **(C)** insulin (0.5 U/kg). **(D)** Serum insulin concentrations before (0-min) and 15 minutes following an intraperitoneal injection of glucose (2g/kg). Statistics are from an ANOVA comparing all groups. All data are mean ±S.E.

3.B. Skeletal muscle membrane cholesterol accumulation and insulin resistance occur within 1 week of high-fat feeding.

We found that mixed hindlimb skeletal muscle membrane from HF-fed mice had a 30-40% increase in cholesterol (Figures 6A and 6B, compare bars 1 and 2). This cholesterol elevation was completely normalized in muscle from the RF and HF +CD groups (Figures 6A and 6B, compare bar 3 to bars 1 and 2).

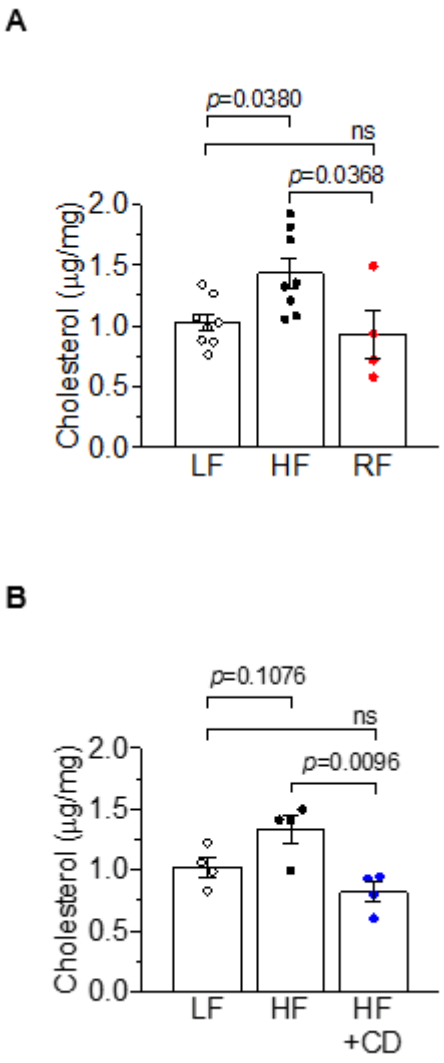


Figure 6. Skeletal muscle membrane cholesterol. (A) Skeletal muscle membrane cholesterol concentrations for LF, HF, and RF groups. **(B)** Skeletal muscle membrane cholesterol concentrations for LF saline, HF saline, and HF +CD group. Statistics are from an ANOVA comparing all groups (n=4-8). All data are mean ±S.E.

3.C. GLUT4 defects

Consistent with a significant insulin-resistant skeletal muscle phenotype, we found that skeletal muscle membrane had nearly a complete loss of insulin stimulated GLUT4 translocation following a 30-minute intraperitoneal glucose challenge (**Figure 7**). This loss was dramatic considering the augmented glucose-stimulated insulin release measured in high-fat-fed animals (see **Figure 3D**). Diet reversal restored insulin stimulated GLUT4 translocation. Studies investigating CD animal GLUT4 translocation are ongoing. Note that we observed no changes in total skeletal muscle GLUT4 content following 1-week of the high fat-feeding challenge as seen by two separate analyses of GLUT4 in whole cell lysates (**Figure 8 A-D**).

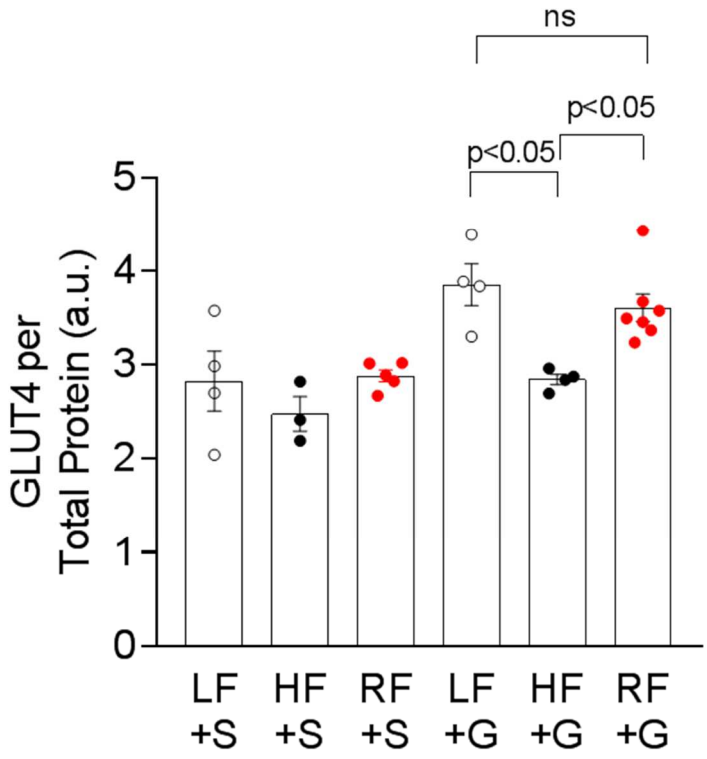


Figure 7. GLUT4 translocation studies in LF, HF, and RF groups show a reversible impairment in glut 4 translocation of HF-fed mice. Quantification of membrane GLUT4 for HF, LF, and RF was performed 30 minutes following *in vivo* glucose stimulation. S = saline; G = Glucose.

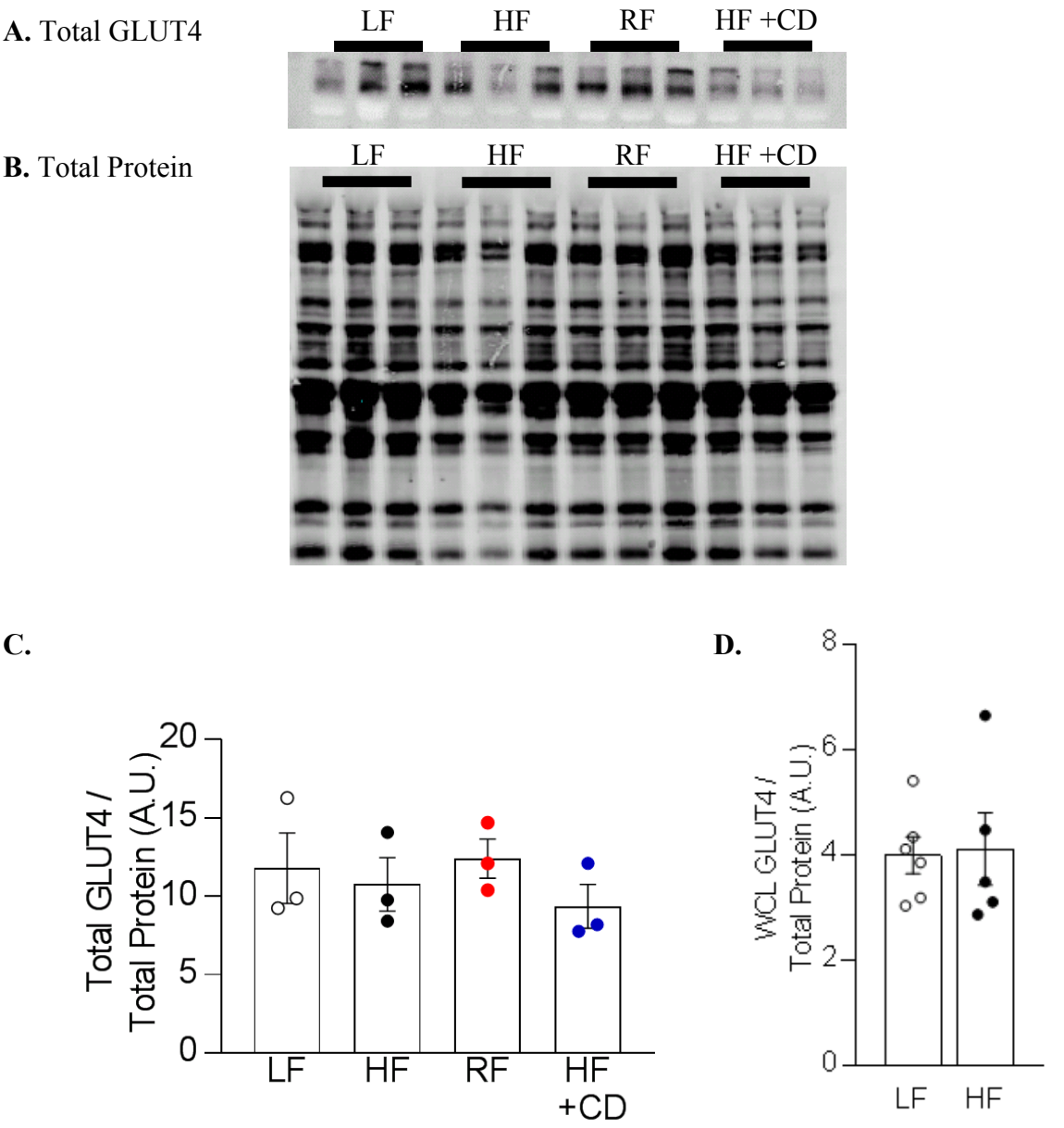


Figure 8. Total cellular GLUT4 content. (A) Representative western blot of total cellular GLUT4 content. **(B)** Total protein bands from the western blot shown in (A). **(C)** Quantification of total cellular GLUT4 for HF, LF, RF, and HF + CD. **(D)** Higher powered examination of the relationship between HF feeding and total cellular GLUT4 content. Statistics are from an ANOVA **(C)** or T-test (n=3-6) **(D)**. All data are mean ±S.E.

3.D. Cortical filamentous actin

Consistent with excess membrane cholesterol compromising cortical F-actin, Figure 9A shows two representative immunofluorescent images of cortical actin filaments in soleus muscle from the LF-, HF-, RF- and HF +CD groups. Qualitatively, a clear loss of cortical F-actin was seen in soleus muscle from the HF group compared to the other groups. For quantification, we scanned the sarcolemma cortical F-actin signal in muscles from three animals per group. Multiple scans were made for each muscle and the individual signal intensity per area are shown for each muscle in Figure 9B. We then averaged all the scans made for a single muscle and present as a single mouse point for each group (see Figure 9C). This analysis showed that muscles from the HF-fed group had a significant reduction in cortical F-actin compared to the other three groups (Figure 9C). We also did this complete analysis in gastrocnemius muscle and found the same association between LF-HF- and RF-fed mice (Figure 8D). We did not collect gastrocnemius muscle from the CD-treated HF-fed mice.

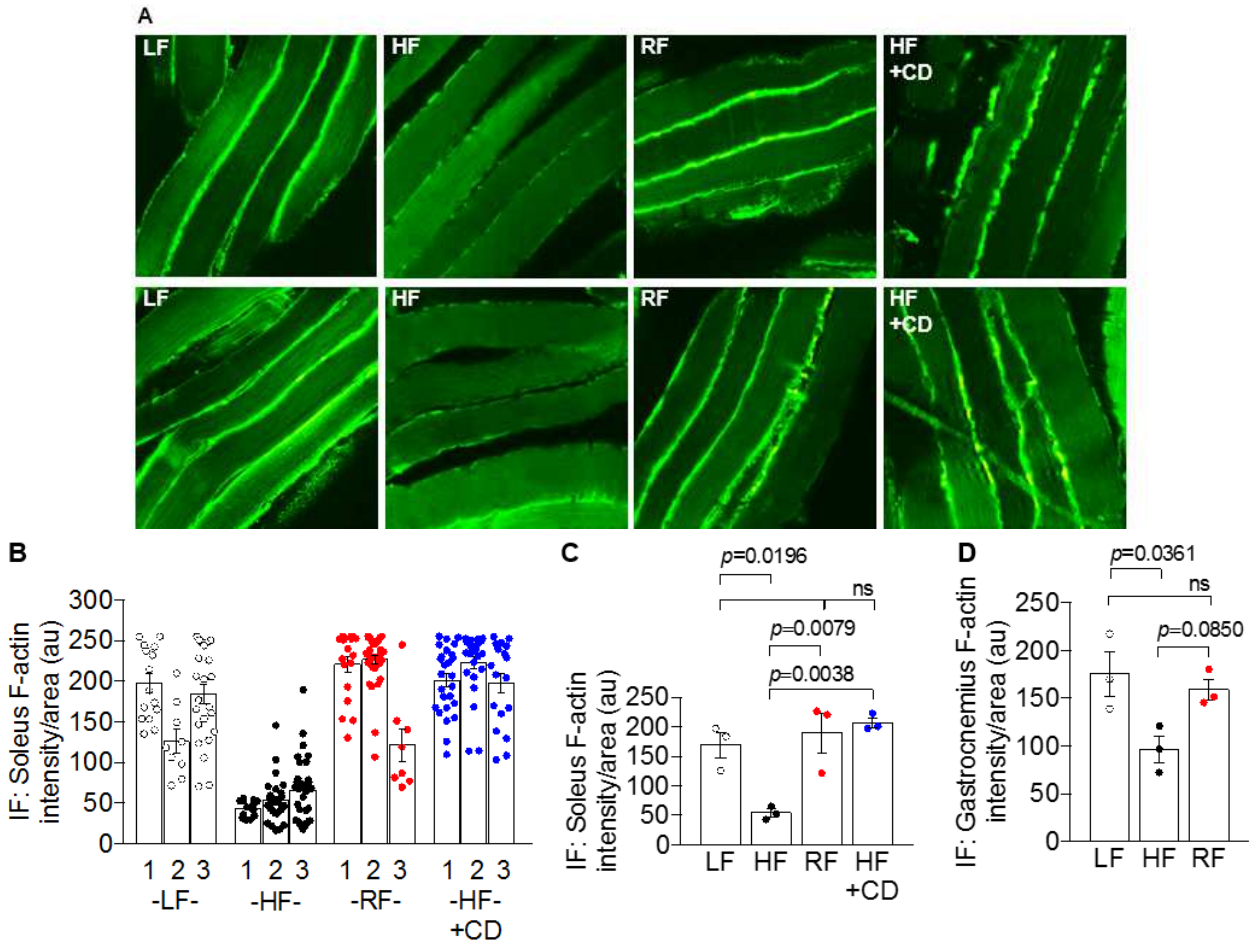

A
LF
HF
RF
HF
+CD
LF
HF
RF
HF
+CD
B
IF: Soleus F-actin
intensity/area (au)
300
250
200
150
100
50
0
1 2 3
-LF-
1 2 3
-HF-
1 2 3
-RF-
1 2 3
-HF-
+CD
C
p=0.0196
ns
p=0.0079
p=0.0038
IF: Soleus F-actin
intensity/area (au)
250
200
150
100
50
0
LF
HF
RF
HF
+CD
D
p=0.0361
ns
p=0.0850
IF: Gastrocnemius F-actin
intensity/area (au)
250
200
150
100
50
0
LF
HF
RF

Figure 9. Skeletal muscle cortical F-actin defects manifest within 1 week of high-fat feeding. (A) Representative images of soleus muscle subjected to immunofluorescent labeling of cortical F-actin. **(B)** Data generated from skeletal muscle cortical F-actin digital image analysis. Each dot represents one scan per mouse and there are three mice per group (LF, HF, RF, or HF +MBCD). **(C)** Quantification and statistical analysis of soleus F-actin data (n=3). **(D)** Quantification and statistical analysis of gastrocnemius F-actin data (n=3). Gastrocnemius data was only available for HF, LF and RF groups. Statistics are from an ANOVA comparing all groups. All data are mean ±S.E.

3.E. Cytokines and insulin signaling

Inflammatory markers have not been observed after 1-week of high fat feeding. We proceeded to confirm this by measuring blood cytokines of diabetogenic inflammatory mediators, tumor necrosis factor alpha, and interleukin 6 which were undetectable in all measured samples. We then quantified gene expression of inflammatory markers tumor necrosis factor alpha and interleukin 6 in skeletal muscle (Figure 10 A and B), adipose tissue (Figure 10 C and D), and the liver (Figure 10 E and F) we observed no change in the gene expression in adipose tissue, liver, or skeletal muscle between LF control mice and 1-week HF-fed mice. Cytokine data was not collected for RF or CD groups since there was no difference between LF and HF fed animals.

Phospho-Akt2 is observably reduced in the skeletal muscle of mice fed a high-fat diet for 1 week. Studies identifying this derangement, however, use a standard dose of insulin for all mice and, yet, we have shown that glucose stimulated endogenous insulin secretion is significantly greater in high-fat fed animals (see Figures 3D, 4D, and 5D). Therefore, the *in vivo* response to a glucose bolus is unclear. Thus, we set out to determine whether insulin signaling impairments were still observed after an endogenous—rather than exogenous—insulin stimulation. Figure11 (A-C) shows quantified western blot data suggesting no impairment in insulin resistance is observed following the intraperitoneal injection of a glucose bolus. This data compliments the cytokine data in suggesting proximal signaling defects may not be mediating early diet-induced insulin resistance.

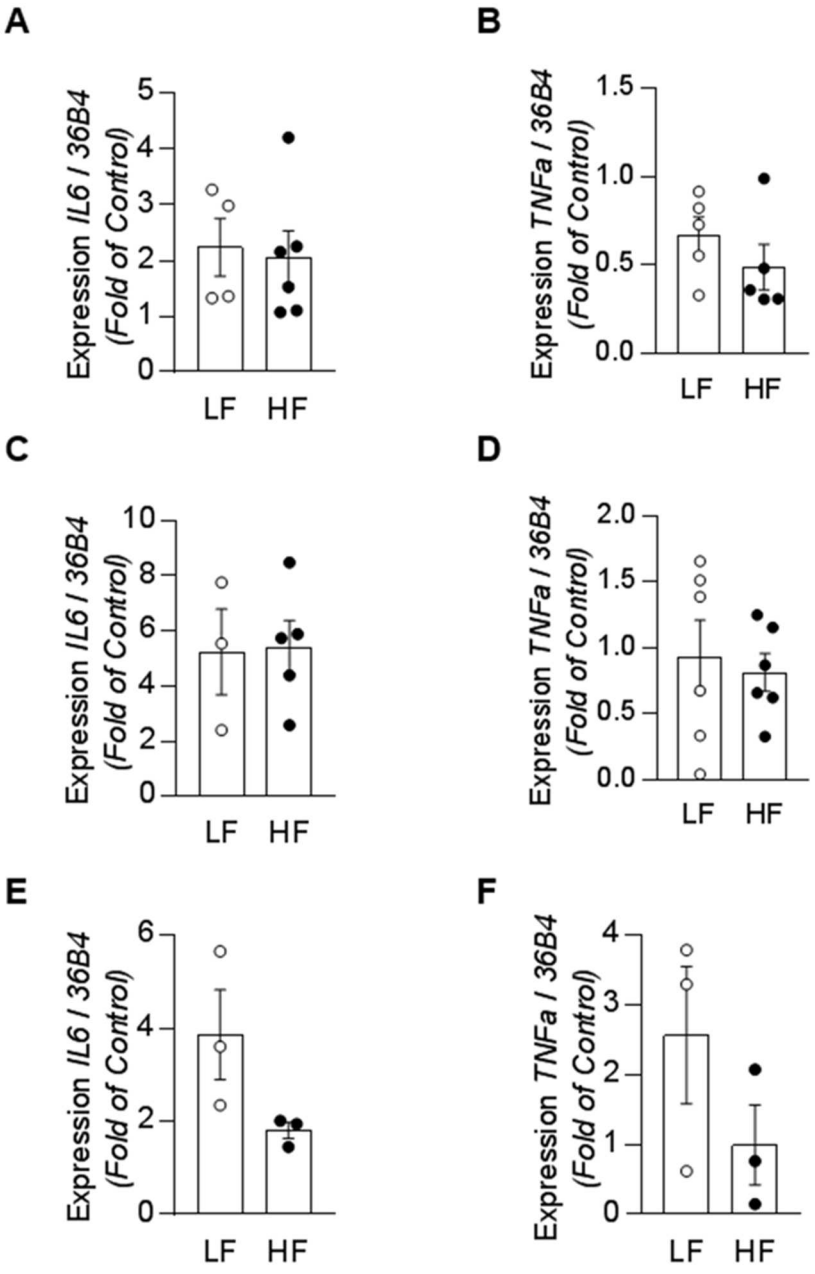
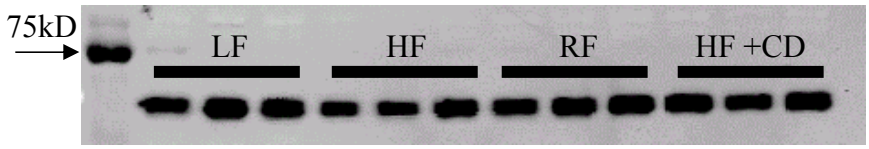
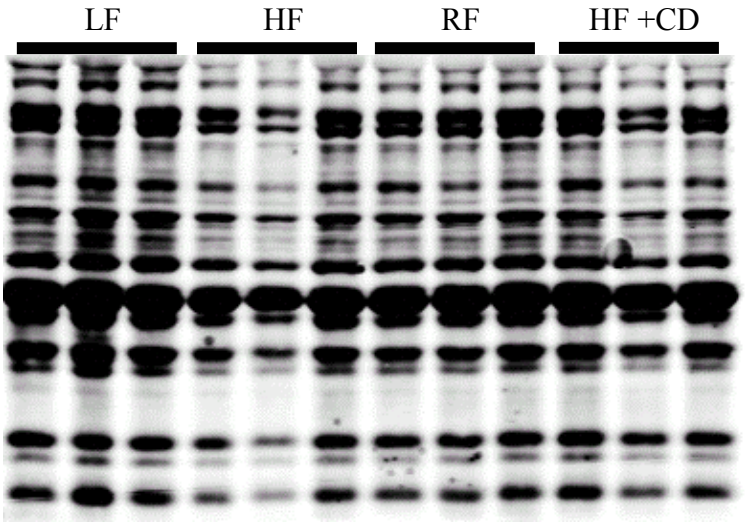


Figure 10. mRNA expression of cytokine genes IL6 and TNFa. (A-B) skeletal muscle gene expression **(C-D)** gene expression in fat and **(E-F)** liver expression. T-test was performed to identify any differences between HF and LF groups. All data are mean ±S.E.

A. pAkt2



B. Total Protein



C.

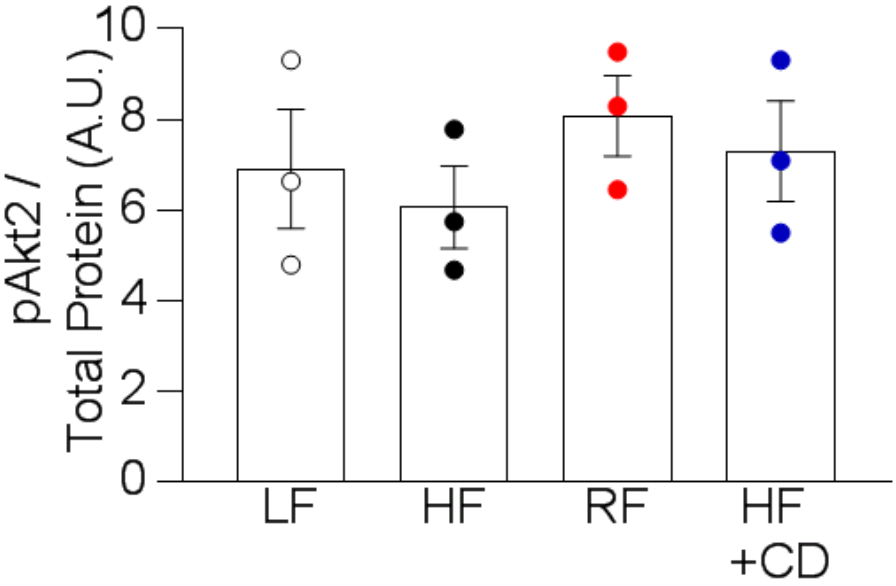


Figure 11. Endogenous-insulin stimulated Akt phosphorylation 15 minutes after a 20% (2g/kg) intraperitoneal glucose injection. (A) Representative western blot of total cellular pAkt2 content. **(B)** Total protein bands from the western blot shown in (A). **(C)** Quantification of total cellular pAkt2 for HF, LF, RF, and HF + CD (n=3). ANOVA was used for statistical analysis of group differences. All data are mean ±S.E.

3.F. Overexpression of skeletal muscle and adipose tissue GFAT impairs glucose tolerance, causes insulin resistance and elevates skeletal muscle membrane cholesterol

Previous work investigating the mechanism of insulin resistance in mice overexpressing muscle and adipose tissue GFAT found a significant reduction in GLUT4 translocation. Studies investigating this GFAT mediated reduction in skeletal muscle GLUT4 translocation is not accompanied by insulin signaling changes. We sought to determine if elevated membrane cholesterol accounted for GFAT induced insulin resistance. In vitro data from our lab would suggest GFAT post-translation regulation of SP1 has downstream effects on cholesterol genes. There was no difference in weight between GFAT transgenic mice and wild type (Figure 12A). Figure 12 recapitulates B) GTT data as seen previously, C) ITT data demonstrates the presence of insulin resistance, and the D) insulin glucose index corroborates the presence of insulin resistance. Figure 12E shows cholesterol data which suggests elevated skeletal muscle membrane cholesterol tends to be elevated in GFAT transgenic mice. Figure 12F shows an upregulated ABCA1 expression trend in transgenic animals suggesting differential cholesterol gene regulation.

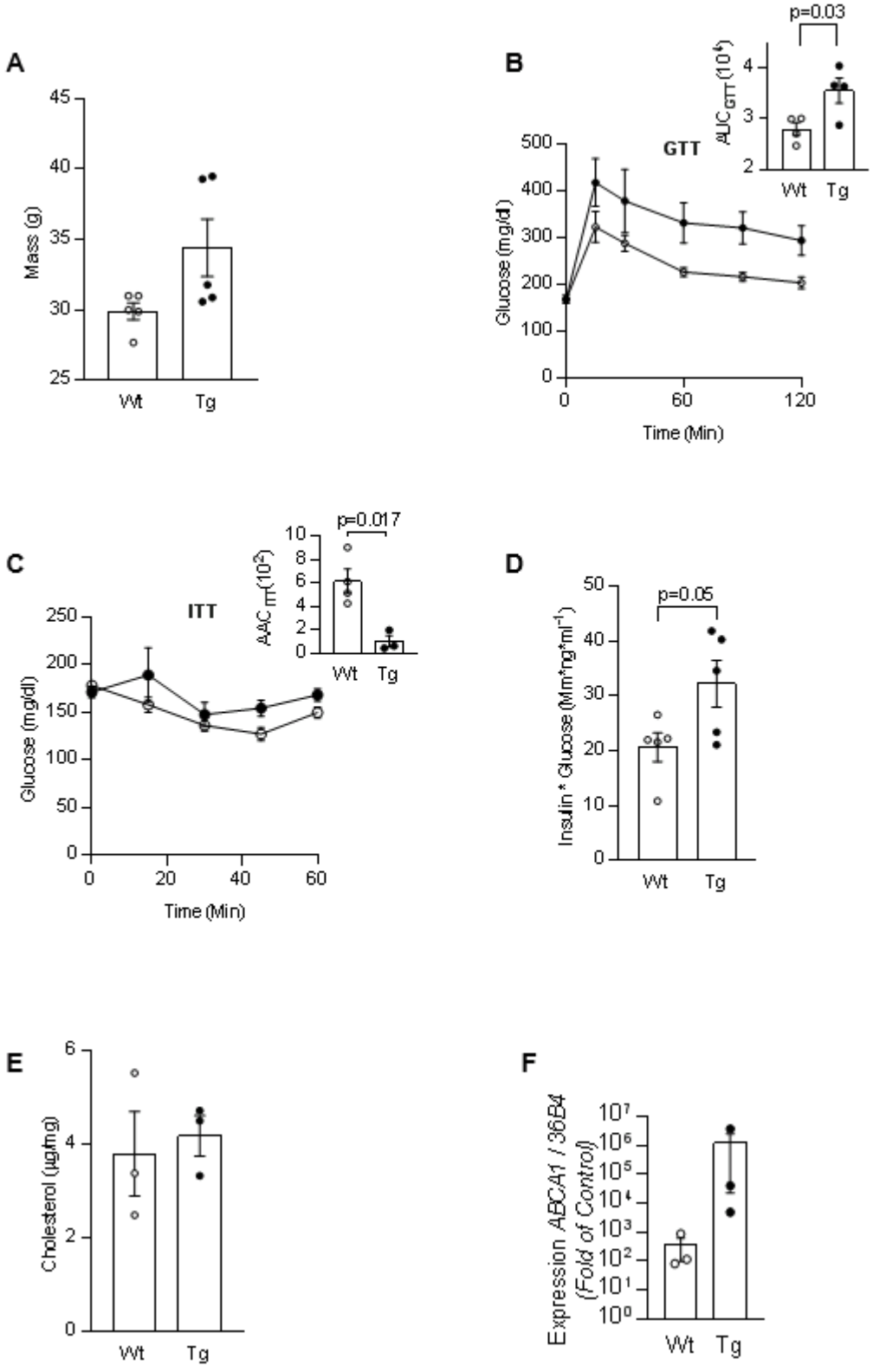

A
Mass (g)
45
40
35
30
25
Wt
Tg
B
GTT
Glucose (mg/dl)
500
400
300
200
100
0
0
60
120
Time (Min)
p=0.03
AUC GTT (10⁴)
4
3
2
Wt
Tg
C
ITT
Glucose (mg/dl)
250
200
150
100
50
0
0
20
40
60
Time (Min)
p=0.017
AAC ITT (10²)
10
8
6
4
2
0
Wt
Tg
D
Insulin * Glucose (Mm*ng*ml⁻¹)
50
40
30
20
10
0
p=0.05
Wt
Tg
E
Cholesterol (µg/mg)
6
4
2
0
Wt
Tg
F
Expression ABCA1 / 36B4
(Fold of Control)
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
Wt
Tg

Figure 12. Transgenic GFAT mice develop glucose intolerance, insulin resistance, and tend to have higher skeletal muscle membrane cholesterol. (A) Body mass **(B)** 2g/kg intrperitoneal administered glucose tolerance test **(C)** 0.5U/kg intraperitoneal injected insulin tolerance test **(D)** Insulin glucose index as a marker of insulin resistance. **(E)** Skeletal muscle membrane cholesterol. **(F)** mRNA expression of *ABCA1* fold change relative to control. More colonies of GFAT mice are currently being bred to validate the mechanisms, elucidated *in vitro*, mediating the causal relationship between overexpression of GFAT and insulin resistance. T-test was performed to identify any differences between wild type and transgenic mouse groups (n=3-5). All data are mean ±S.E.

3.G. SP1 inhibitor mithramycin prevents diet induced glucose intolerance and does not alter food intake or weight.

Cell culture studies in our lab have previously shown a palmitate induced increase in o-linked glycosylation of the transcription factor Sp1. This post-translational modification promoted Sp1 binding to the promoter region of HMGR resulting in elevated cellular HMGR protein levels. Hyperinsulinemia, hyperlipidemia, and hyperglycemia all independently increase plasma membrane cholesterol *in vitro* through the HBP. Studies blocking HBP upregulating or inhibiting Sp1 binding to DNA prevent HBP facilitated plasma membrane cholesterol accumulation. We tested the association between Sp1 inhibition, insulin sensitivity and membrane cholesterol *in vivo* by injecting mice with mithramycin. Mithramycin prevented glucose intolerance (Figure 13A), insulin resistance (Figure 13B), and mitigates membrane cholesterol accumulation (Figure 13C). This does not appear to be a consequence of inhibited food intake as mice treated with Sp1 consumed the same amount of calories as their high fat counterparts (Figure 13D) and had no change in weight (Figure 13E). A second round of studies are ongoing with larger sample sizes to validate the caloric intake and weight trajectories findings.

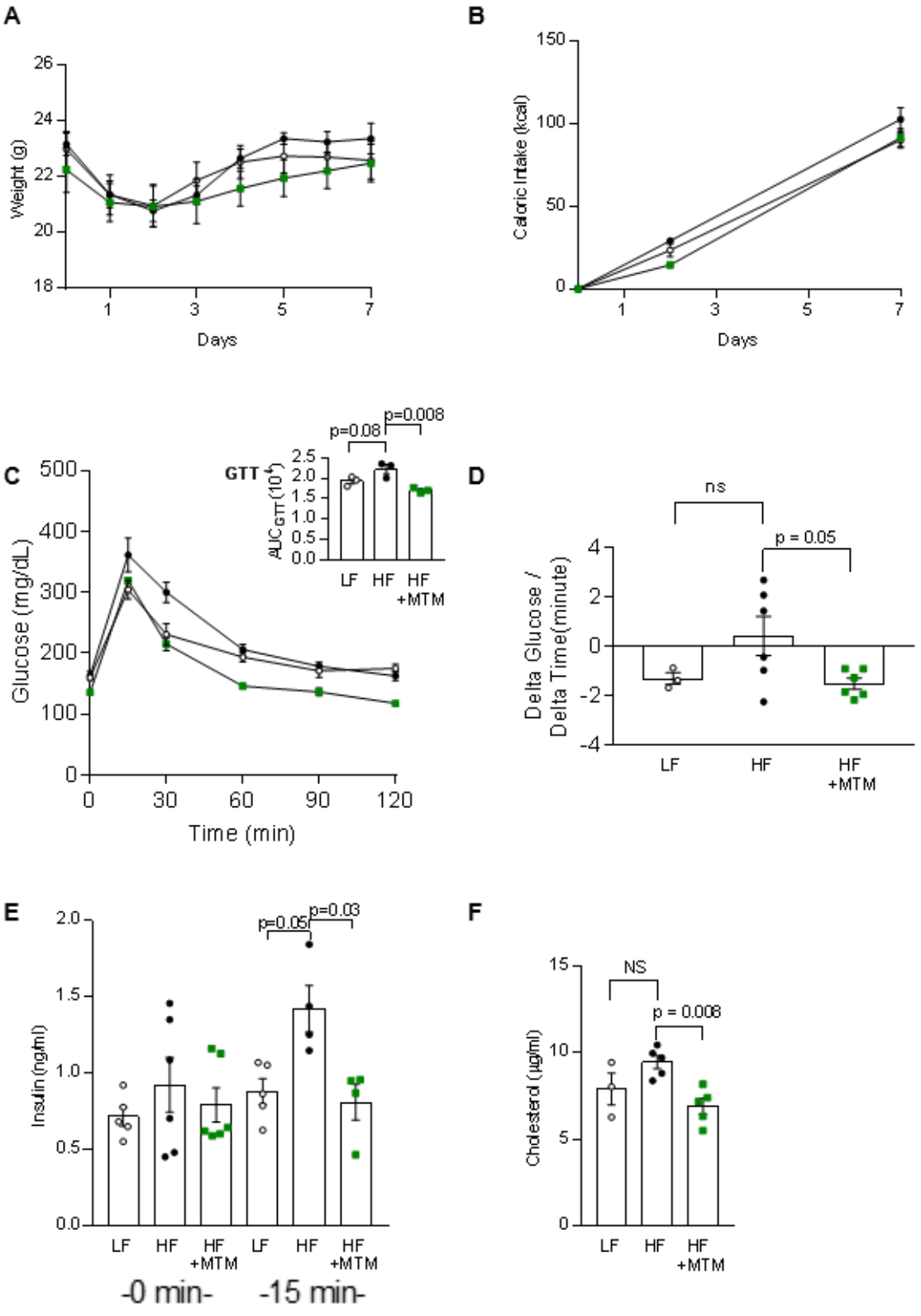

A
Weight (g)
Days
B
Caloric Intake (kcal)
Days
C
Glucose (mg/dL)
Time (min)
GTT
AUC GTT (10^4)
p=0.08
p=0.008
LF
HF
HF +MTM
D
Delta Glucose / Delta Time(minute)
ns
p = 0.05
LF
HF
HF +MTM
E
Insulin (ng/ml)
p=0.05
p=0.03
LF
HF
HF +MTM
-0 min-
-15 min-
F
Chdesterol (µg/ml)
NS
p = 0.008
LF
HF
HF +MTM

Figure 13. Mithramycin injections did not affect weight or appetite and prevented glucose and insulin intolerance in 1-week high-fat-fed mice. (A) Weight trajectories over the seven-day study period. **(B)** Total caloric intake at days 2 and 7. **(C)** Glucose tolerance test (2g/kg intraperitoneal injection) **(A-C)** Closed circles are HF saline treated controls, open circles are LF saline treated controls, and green squares are HF animals injected with (mithramycin) HF +MTM experimental mice. **(D)** Glucose slope over the first 30-minutes of an intraperitoneal injected (0.5 U/kg) insulin tolerance test. **(E)** Insulin values at baseline (0-minutes) and 15-minutes following a (2g/kg) intraperitoneal injection of glucose. **(F)** Skeletal muscle membrane cholesterol from mixed hindlimbs excised from LF saline, HF saline, and HF +MTM experimental mice. ANOVA was used to test for differences between groups (n=3-6). All data are mean ±S.E.

3.H. Cholesterol laden methyl-β-cyclodextrin injections causes glucose intolerance and 1-week high fat diet increases HMGR expression.

Strengthening the causal relationship between membrane cholesterol accumulation and glucose intolerance are experiments whereby shuttling cholesterol into cells can induce insulin resistance in GLUT4 translocation. This corresponds nicely with data we discovered showing the upregulation of *Hmgr* expression (Figure 14A) is upregulated in 1-week high-fat-fed mice suggesting *de novo* cholesterol synthesis is contributing to elevated membrane cholesterol in diet-induced insulin resistant mice. Future studies are underway with the aim of parsing out the differential regulation of cholesterol genes after 1-week of HF feeding. We will determine which cholesterol genes are important in the early regulation of skeletal muscle membrane cholesterol.

To test the concept of membrane cholesterol accumulation *in vivo*, we used two doses of cholesterol laden methyl-β-cyclodextrin (8:1 molar ratio) to acutely induce glucose intolerance in chow fed (Figure 14B). We observed a 10% increase in the glucose are under the curve of mice injected with cholesterol laden methyl-β-cyclodextrin relative to their saline-control chow-fed counterparts. Further examination of acutely induced insulin resistance in chow fed mice is currently underway. We are optimizing the cholesterol-to-methyl-β-cyclodextrin molar ratio since 8:1 only facilitates a modest increase in membrane cholesterol *in vivo*. We think the more potent molar ratio of 4:1 will induce a larger glucose intolerance. We will then determine whether the cholesterol addition impairs GLUT4 translocation and insulin signaling.

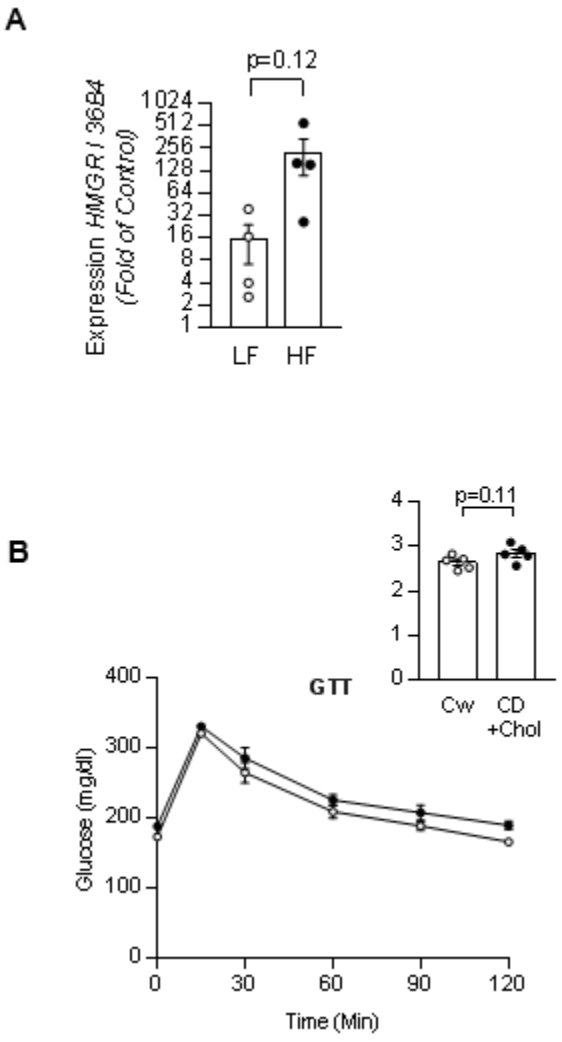


Figure 14. High-fat-feeding increases HMGR expression and exogenous cholesterol injections acutely impairs glucose tolerance in chow fed mice. (A) Fold change of *HMGR* expression over control in low-fat-fed and high-fat-fed mice. **(B)** Glucose tolerance test (2g/kg intraperitoneal injection) 5 hours after two injections of (0.3 ml) of 5 mM methyl-β-cyclodextrin laden with cholesterol at an 8:1 (methyl-β-cyclodextrin:cholesterol) molar ratio. T-test was used for statistical testing (N=4-6). All data are mean ±S.E.

Chapter 4. Discussion

Skeletal muscle membrane cholesterol accumulation and F-actin cytoskeletal derangements in mice fed a high-fat diet for 1 week are causally associated with glucose intolerance and insulin resistance. This is the first mechanism of early insulin resistance ever identified that negatively affects GLUT4 regulation in the initial stages of Western-style high-fat feeding challenge. The observed membrane/cytoskeletal derangements were restored to normal when the high-fat diet was removed or methyl-β-cyclodextrin was used to remove cholesterol from skeletal muscle membranes.

Causality for excess membrane cholesterol impairing glucose tolerance was further supported by the acute diabetogenic effect of delivering exogenous cholesterol into chow fed mice. Analyses of tissue from these mice injected with cholesterol-laden methyl-β-cyclodextrin are currently underway, including GLUT4 translocation determination. The effect of cholesterol addition to membranes will be telling and should further clarify the relationship between membrane cholesterol and insulin signaling defects.

Previous cell culture studies in the lab found excess membrane cholesterol was caused by HBP-mediated increase in the O-GlcNAc post-transcriptional modification to Sp1. Use of the Sp1 inhibitor mithramycin prevented diet-induced insulin resistance in high-fat fed mice and trended to reduce membrane cholesterol concentrations. Follow-up studies are underway to determine whether Sp1 inhibition improves GLUT4 translocation in high-fat fed mice. Findings presented show a glucose intolerance in GFAT transgenic mice, and skeletal muscle cholesterol appears to be elevated in skeletal muscle membrane of the GFAT mice. Future research will also importantly evaluate whether mithramycin is sufficient for preventing insulin resistance in GFAT transgenic mice.

There are a lot of mediators of insulin resistance, but when broken down to their mechanisms of action, many negatively impact the same proteins along the proximal insulin-signaling pathway. These signaling pathway proteins are common in liver, muscle, and fat. Therefore, we would expect insulin resistance to occur simultaneously in all three tissues. This, however, is not the case in animals or humans. Cardiac and skeletal muscle, and adipose tissue glucose uptake approaches maximum impairment shortly following high fat feeding; whereas, suppression of hepatic glucose output by insulin takes many more weeks to be maximally defective resulting in increased hepatic glucose production[91].

In humans, tissue specific insulin sensitivity differences define many at risk for type 2 diabetes[5-8]. Mutations in *AKT2* found in a Finish population with congenital fat, skeletal muscle, and hepatic insulin resistance is instructive when considering the distribution of IFG, IGT, and combined IFG/IGT in the general population. The carriers of the *AKT2* mutation, present with hepatic and skeletal muscle insulin resistance[152]. This presentation suggests a mechanism of insulin resistance in the insulin-signaling cascade (Insulin Receptor→Akt2) would manifest in a patient with combined IFG/IGT[152]. Although defective insulin signaling to Akt does not represent a major node of insulin resistance in skeletal muscle, this may not be the case for the liver; i.e., impairment in Akt signaling in the liver may be a critical aspect of hepatic insulin resistance. It is recognized that IRS1 inhibition causes both hepatic and skeletal muscle insulin resistance, but it is possible that IRS1 inhibition in humans can be compensated for through hepatic expression of IRS2 resulting in isolated IGT. There is little evidence in humans supporting a role for IRS1 mutations since humans with mutations have a mild or

no insulin resistance and little to no increased risk for type 2 diabetes[153]. Moreover, people with IRS1 mutations can also have elevated fasting glucose [154, 155]and, while IRS1 is generally considered the GLUT4 regulating isoform, people with IRS2 mutations have both a hepatic and skeletal muscle insulin resistance association with body fat longitudinally[156]. Therefore, it is just as plausible that insulin resistance in isolated IGT lies somewhere distal to Akt2. Complimenting this research is the literature I cited in the introduction, where bypassing IRS, PI3K and Akt demonstrated the continued presence of insulin resistance.

The presentation of isolated skeletal muscle insulin resistance in a sizeable proportion of the population suggests a mechanism precluding the insulin signaling cascade exists that warrants investigation to understand skeletal muscle insulin resistance underlying IGT. Distal to Akt2, a mutation in AS160 has been discovered and, as expected, is associated with skeletal muscle insulin resistance. This mutation is a gain of function mutation that releases GSVs allowing them to translocate to the membrane. Nonetheless, this mutation that causes "unregulated" GLUT4 positioning in the plasma membrane likely elicits skeletal muscle glucose toxicity, perhaps HBP-mediated cholesterol accumulation. In line with this thought, the AS160 mutation and unregulated GLUT4 translocation is associated with post-prandial hyperglycemia and fasting hypoglycemia. Generally, however AS160 mutations are rare and there is no evidence for widespread AS160 downregulation in early insulin resistance[157]. Rather, as mentioned earlier, skeletal muscle insulin resistance is still present when AS160 is circumvented, and Akt2 downregulation does not necessarily result in AS160 downregulation, despite

impaired GLUT4 translocation. More so, Akt-independent pathways, bypassing AS160 are sufficient in producing GLUT4 translocation to the membrane.

Insulin-stimulated glucose disposal is maximally impaired prior to the onset of IGT[158], yet, weight gain is still ongoing in persons developing IGT which suggests proximal insulin signaling is still intact, at least in adipocytes[158]. Populations with isolated IGT tend to be fatter and older than populations with IFG so it is possible that skeletal muscle insulin resistance is not a result of a defect in insulin signaling proteins, but rather the result of a normal physiologic response to high caloric intake[137]. As my dissertation research has shown, along with previous studies, skeletal muscle insulin resistance is a consequence of HBP overactivity[78]. Generally considered a nutrient sensor, HBP regulation of genes responsible for cholesterol handling may be a normal and necessary aspect of cholesterol metabolism meriting further investigation. Consequently, obesity-mediated cholesterol gene regulation may lead to skeletal muscle insulin resistance. Ding et al. demonstrated this very association between obesity regulation of cholesterol genes and type 2 diabetes risk[110]. The cholesterol efflux protein, ABCG1, is also often seen to be epigenetically modified in obesity and epigenetic modification to ABCG1 is frequently found as a risk factor for type 2 diabetes[112, 117-120, 159].

The relationship between cholesterol transport into and out of muscle and fat will be important for understanding the mediation of cellular cholesterol accumulation and GLUT4 dysregulation. I mentioned earlier that data suggests increased skeletal muscle GFAT activity in isolation may be insufficient in producing skeletal muscle insulin resistance. Rather, both skeletal muscle and adipose tissue GFAT overexpression is

required for the onset of skeletal muscle insulin resistance. We identified the upregulation of HMGR in response to HBP activity[78], but HMGR activity alone may not be sufficient *in vivo* to cause cellular cholesterol accumulation. *In vivo*, cellular cholesterol influx and efflux may be as important for regulating cholesterol levels as *de novo* synthesis. To study the relationship, it would be important to investigate the effect LDLR, VLDLR, ABCA1, and ABCG1 gene knockout has on metabolism in chow fed and high fat fed mice. It will be equally important to determine the effects of Sp1 regulation on HMGR. Does a skeletal muscle specific knockout of the Sp1-promoting region in HMGR protect against diet-induced insulin resistance? Does whole body knockout of this same region prevent against diet-induced insulin resistance?

The transience of cholesterol accumulation is another aspect that will be important to investigate. Hyperglycemia for a short period of time has been shown to induce skeletal muscle insulin resistance, but not through the HBP-meditated post-translational regulation of proteins[88]. Another nutrient sensor implicated in insulin resistance is AMP-activated protein kinase (AMPK). Responsive to levels of adenosine monophosphate and ATP, AMPK allosterically regulates many anabolic and catabolic proteins. One such protein, is HMGR, which has an AMPK responsive site (Serine 872), that, once phosphorylated, deactivates HMGR activity[160]. Under hyperglycemic conditions, AMPK would be inactivated and HMGR would be functional, allowing for cellular cholesterol accumulation to commence. Knock-in mutation of the serine 872 codon in skeletal muscle would be expected to cause unregulated HMGR activity and skeletal muscle insulin resistance. Furthermore, constitutive activation of AMPK should also prevent HMGR-mediated cholesterol accumulation and can be achieved through

either gene technology or using a drug such as AICAR. AICAR has been demonstrated to improve insulin resistance[161] and *in vitro* studies of GLUT4 translocation demonstrate that this improvement can be mitigated by adding cholesterol-laden methyl-β-cyclodextrin to culture media[162], thereby increasing membrane cholesterol concentrations.

Aside from HMGR are proteins that regulate cholesterol transport—APOA1, ABCA1, ABCG1, and the LDLR—have all been identified as meditators of insulin resistance. Preliminary studies by our lab have shown that ABCA1 knockout in skeletal muscle can cause a mild glucose intolerance (see Figure 15). This phenotype, however, was mild and not what we expected. We identified possible upregulation of ABCG1 and fat regulation of cholesterol efflux as potential confounding factors in these ABCA1 experiments. Mice with skeletal muscle specific double knockout of ABCA1 and ABCG1 are currently in development, but the role of fat on skeletal muscle insulin sensitivity is still in need of investigation.

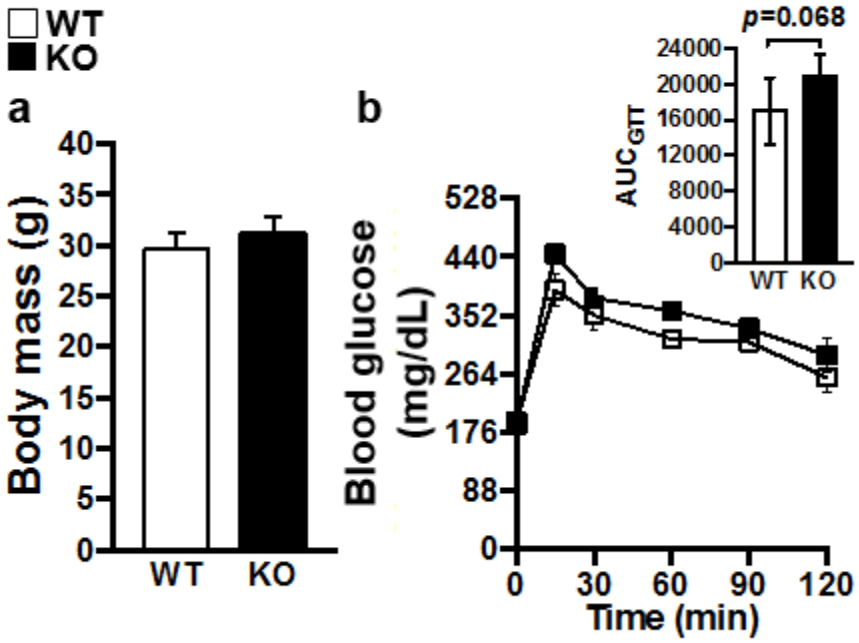


Figure 15. Body mass and glucose tolerance test for skeletal muscle specific ABCA1$^{-/-}$ knockout mice.

Adipose tissue HBP overactivation is sufficient to cause a mild skeletal muscle insulin resistance in otherwise healthy animals[137]. Regulation of skeletal muscle insulin resistance by adipocytes is complex as there are many endocrine and paracrine signals produced by fat with the ability to prompt insulin resistance. Inflammatory markers, for example, are important signals synthesized in adipose tissue and capable of propagating insulin resistance. Inflammation onset, however, is after the onset of insulin resistance in diet-induced insulin resistance and adipose-specific HBP-mediated insulin resistance does not appear to be mediated by inflammation[137]. Overactivity of HBP in fat has been shown to decrease circulating adiponectin levels, a hormone that would be expected to inhibit HMGR activity by it stimulation of AMPK and may also regulate cholesterol efflux[137, 163]. Adiponectin concentrations, moreover, have been observed to decrease early in diet-induced insulin resistance[91].

Cholesterol efflux regulation by adiponectin has been observed *in vitro* and *in vivo*. Adiponectin decreases the rate of ApoA1 degradation in humans and increases ApoA1, ABCA1, and SR-BI concentrations in HepG2 cells[164-166]. The effect that adiponectin has on the cholesterol content of skeletal muscle is unknown. Metabolically healthy fat may counteract the deleterious effects of HBP upregulation in muscle, through the secretion of adiponectin and upregulation of cholesterol efflux. Conversely, decreased adiponectin secretion from metabolically unhealthy fat may result in an adiponectin loss of AMPK activity suppressing HMGR activity and a decreased cholesterol efflux capacity and thus result in an increased skeletal muscle membrane cholesterol. Interestingly, ApoA1 mimetics have recently been shown to improve insulin sensitivity and, hence, provide a strong basis for investigating cholesterol efflux as a mechanism by

which fat, through adiponectin signaling, can influence skeletal muscle insulin sensitivity[133, 167].

Mechanistically, research aimed at understanding the effect membrane cholesterol has on GLUT4 translocation should be parsed out further. Previous studies in our lab have identified a key role for PIP2 and actin regulation, but understanding the relationship between plasma membrane cholesterol domains, PIP2 concentrations, and actin assembly merits future investigation. Many of the steps involved in insulin-stimulated GLUT4 translocation occur at the plasma membrane, and therefore a relationship between membrane derangements, aPKC defects, translocation proteins, calcium channels, calcium influx and PLD1 associated membrane priming events would be valuable in further delineating the mechanism by which excess membrane cholesterol impairs insulin stimulated GLUT4 translocation.

Chapter 5. Conclusion

These studies suggest elevated skeletal muscle membrane cholesterol occurs early in the etiology of diet-induced insulin resistance. Encouragingly, these data also show that membrane cholesterol is mediated by nutrient excess and is reversible. Moreover, reversal of diet or removal of cholesterol was sufficient to restore glucose tolerance in high-fat-fed animals. The addition of cholesterol was also sufficient for inducing glucose intolerance. Diet-induced insulin resistance was prevented in mice administered mithramycin suggesting a significant role for Sp1-mediated transcription in the development of glucose intolerance. These studies add to the sizeable evidence that diet-induced insulin resistance in some part is mediated by a cytoskeletal and membrane mechanism distal to proximal insulin signaling.

Chapter 6. Appendix

Pre-clinical scientists are required to quantify data using specialized measurement tools for a manuscript to be acceptable for publication. Quantification and statistical analysis of data may not be the best choice for interpreting finds—instead consider repeatability of experiments as a measure of significance—but regardless quantification is required. Lysates we use for data analysis are products of tissues with differing weights and pellets of assorted sizes. This results in sample concentration variance which needs to be accounted for in data analysis. We use total protein as a measure of cellular material for any given sample. Usually, data are standardized using a per-protein standard (measure/tot protein) this procedure is simple in its math and the per-protein denotation makes intuitive sense. This language though is deceptive.

The use of ratios has been discussed thoroughly in epidemiologic literature among other fields[168-170]. These studies focus on the finding of spurious correlations when a variable is divided by another variable (i.e. exercise/weight). When this new ratio is used for correlation analysis with another variable (i.e. glucose) we discover a biased result, that is, this finding is mostly influenced by the correlation between weight and glucose, resulting in biased findings.

The scenario provided above is not the scenario we are dealing with for standardization of molecular biology measures. In these experiments, we are using total protein as an indicator of cellular material in lysate, a random variable, and the division should not cause spurious associations. There is a different issue when the per-protein standard is used, error. For example, western blots are standardized to abundant proteins or preferably to total protein using a ratio[171]. Gilida et al. demonstrate the superiority of a

per-protein standard using total protein rather than abundant proteins (GDPH), and Kevin Janes confirmed these findings and expanded on the work. In his manuscript, he discusses the importance of a zero-intercept (removing background) from the western blot quantification[171, 172]. Unfortunately, a zero-intercept requirement is widely unknown and getting accurate measurements of background to achieve zero is statistically impossible, although an acceptable range near zero will produce satisfactory results[172]. The discussion by Kevin Janes and others has identified the flaws with the use of ratios but fall short of suggesting regression analysis[172-175].

Considering the potential for standardization to bias our results I decided to determine the appropriateness of regression-based analysis for statistical analysis of variables normally standardized using a per-protein technique. The number of samples-to-variables in the regression analysis is a concern since a high proportion of samples-to-variables can inflate significance, resulting in type 1 error, traditionally an acceptable proportion is 1:20[176]. Our goal, however, is to remove error incorporated into our data by the variance in sample concentration. For this purpose, we would like to calculate the residual of the variable (Y) to the predict measurement value (Observed value – predicted value) for the equation $Y = B0 + B1X1+\ldots+ e$, where X1 is total protein and Y is our measurement of interest. Significance in this case is not of interest but rather the accuracy of parameters B0 and B1, which, according to Austin and Steyerberg, can be accurately calculated (within a 10% error rate) when there are as few as 2 samples per variable, with the caveat that this is rule applies to large datasets[177]. The residuals from this model would be analyzed by a t-test to determine the group differences between experimental and control conditions.

I proceeded to run statistical simulations to test the sensitivity and specificity of various standardization methods with the aim to identify the most appropriate method for data analysis. Methods of data analysis used were per-protein standard, residual, and raw unaltered data. By varying the number of samples (n), intercept, diet effect size, protein effect size, and error distributions we could generate models predicting the sensitivity of a t-test based on these factors.

The purpose for running this simulation was to establish the method that is most sensitive to experimental group differences. There was no difference in test specificity among the three methods. I did see an expected increase in sensitivity for all methods when n is increased, the effect size is large, or the error distribution is decreased. Figure A-16 is a representative graph generated from a simulation in which the intercept was varied while the other variables were held constant. Shown in the graph is the effect the intercept has on the sensitivity of a t-test to discriminant between treatment and control groups, each color represents a method of adjustment described above. Of note, is the exponential decrease in the sensitivity of the per-protein method as the intercept moves further away from zero. It is also important to note that the residual method was consistently sensitive regardless of deviation from intercept. The unadjusted data was most sensitive to data with a large experimental effect size and/or a small error distribution. Overall these simulation studies establish the per-protein standard to be an unreliable method for detecting small-to-medium effect sizes when the intercept varies from zero. In most studies, the intercept will be a random variable centered around zero. Future work will examine: 1) the probability of an intercept to fall within an acceptable range for detecting small effect sizes, 2) the concordance between parameters and their

estimates from linear regression in small sample sizes 3) the accuracy of a t-test in determining experimental effect size from residuals 4) the potential for GLM modeling of ANCOVA for adjusting data and 5) the best method for handling data with batch effects (ANCOVA or t-test of Residuals). The most important takeaway from this data is that the residual method is superior to the per-protein method and is the most reliable method for detecting differences between experimental groups.

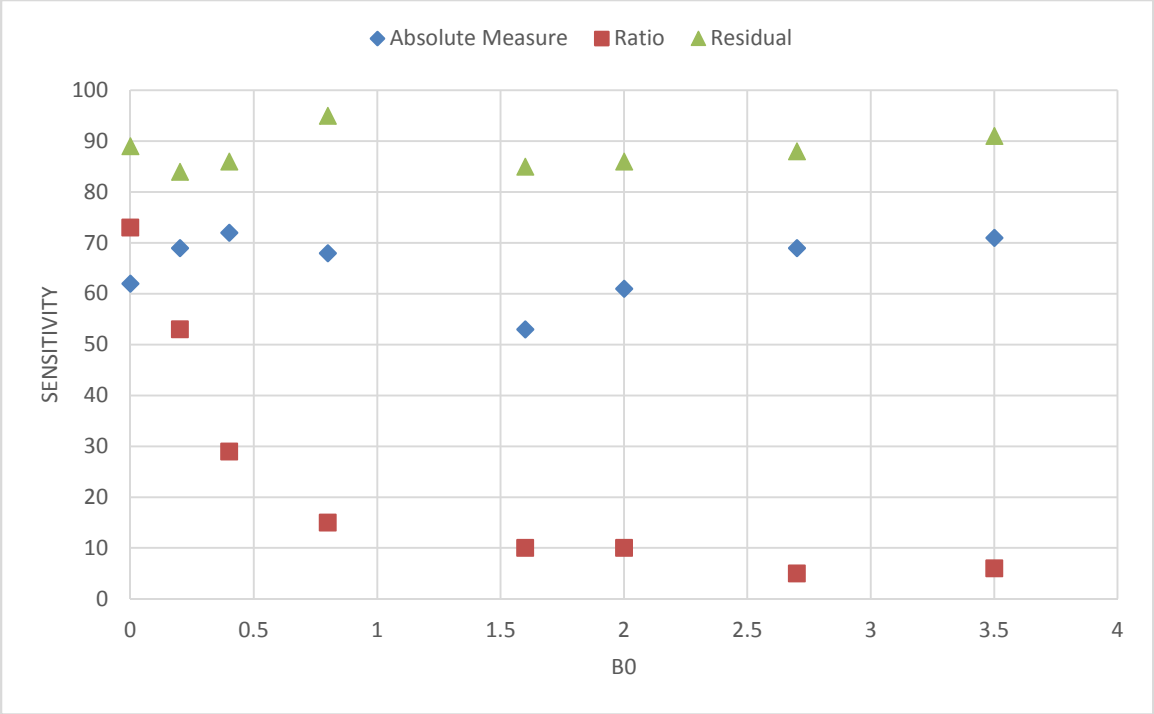


Figure A-16. Sensitivity of data adjustment methods. B0 by Sensitivity, B1=0.3, Std Error=0.2, n=13, Effect Size=0.5

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Chapter 8. Curriculum vitae

BRIAN A. GRICE

Education and Training

1. Ph.D. Cellular and Integrative Physiology (2019) Indiana University degree, earned at IUPUI
2. Indiana University School of Medicine MSTP student. MS3 student. Expected graduation 2021.
3. Bachelor of Science in Health Science (2009), Benedictine University, Lisle, Illinois
4. National Institutes of Health Grant Writing 101 & 102 Workshop Series November 2011
5. Statistical Analysis Software Basic Programming Course NIH 2010
6. NIH epidemiology study design and analysis weekly seminar on topics in *Modern Epidemiology* by Robbins, Greenland, and Lash 2010-2014

Awards

1. *Phi Theta Kappa* Scholarship Recipient (2006-2009)
2. Scholl Foundation Research Award (2008)
3. Intramural Research Training Award, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Phoenix, AZ (2010–2013)
4. Indiana University Fellowship (2014)
5. Indiana University Diabetes and Obesity Training Program (T32DK064466)
6. Indiana University Medical Scientist Training Program (T32GM077229)

Experience

1. NIDDK, Phoenix, Arizona, Intramural Research Training Award Fellow, 2010-2013
2. NIH, special volunteer position 2013-2019
3. Statistical Analysis Software programmer, 2010-2019
4. Data manager with the Lifestyle Intervention for Expectant Moms (LIFE-Moms) study, 2013-2019.

Research Interests

1. Insulin resistance physiology
2. Diabetes clinical trials
3. Diabetes genetics
4. Diabetes physiology
5. Statistical methodology and epidemiologic study design

Grants

1. NIDDK Diabetes training grant T32DK064466
2. NIGMS training grant T32GM077229

Manuscripts

1. **Grice BA**, Tackett L., Elmendorf JS. Membrane Cholesterol Accumulation is a Causal Mechanism of Skeletal Muscle Insulin Resistance Mediated by Hexosamine Biosynthesis Pathway Post-Translational Modifications to SP1. In Preparation
2. **Grice BA**, Barton KJ, Tackett L, et al. Excess Membrane Cholesterol is an Early Contributing Reversible Aspect of Skeletal Muscle Insulin Resistance in

C57BL/6NJ Mice Fed A Western-Style High-Fat Diet. In Press. AJP-Endocrinology and Metabolism

3. Horbal SR, **Grice BA**, Evans A, et al. Trial to Evaluate and Improve Interprofessional Practice Behaviors at a Student-Run Free Clinic. In Press. Journal of Interprofessional Education & Practice.
4. Hohenadel MG, Thearle MS, **Grice BA**, et al. Brain-derived neurotrophic factor in human subjects with function-altering melanocortin-4 receptor variants. *Int J Obes (Lond)* **38**(8): 1068-1074.
5. **Grice BA**, Mason CC, Weil EJ, et al. The relationship between insulin sensitivity and maximal oxygen uptake is confounded by method of adjustment for body composition. *Diabetes Vasc. Dis. Res. Off. J. Int. Soc. Diabetes Vasc. Dis.* 10:530–535 2013.
6. **Grice BA**, Nelson RG, Williams DE, et al. Associations between persistent organic pollutants, type 2 diabetes, diabetic nephropathy and mortality. *Occup Environ Med*. 74(7):521-527, 2017.
7. **Grice BA**, Elmendorf JS. New aspects of cellular cholesterol regulation on blood glucose control-review and perspective on the impact of statin medications on metabolic health. *US Endocrinology*. 13 (2): 63-68 2017.

Abstracts/Presentations

1. **Grice BA**, Delaney L, Tischler M. Characterization and Analysis of Antibiotic Resistance in Bacteria Isolated from Resident Canada Geese and Goslings. Oral Presentation at: Nineteenth Annual Argonne Symposium for Undergraduates in Science, Engineering and Mathematics; 2008 November 7-8; Argonne, IL.

2. **Grice BA**, Mason CC, Weil EJ, Curtis JM, Knowler WC, Pomeroy J. The Relationship between Maximal Oxygen Uptake and Plasma Insulin Concentration Depends on Method of Body Size Adjustment. Poster session: American Diabetes Association 71st Scientific Sessions; 2011 June 24-28; San Diego, CA.
3. **Grice BA**, Nelson RG, Knowler WC, Mason CC, Bullard KM, Williams DE, Gehle K, Turner WE, Pavkov ME. Associations between persistent organic pollutants and type 2 diabetes. Poster session: American Diabetes Association 72nd Scientific Sessions; 2012 June 8-12; Philadelphia, PA.
4. Pavkov ME, **Grice BA**, Knowler WC, Mason CC, Williams DE, Turner W, Nelson RG. Persistent organic pollutants (POPs) and ESRD or death in type 2 diabetes. 25th European Diabetic Study Group Meeting; 2012 May 17-19; Dublin, Ireland.
5. **Grice BA**, Hohenadel M, Mason CC, Krakoff J, Williams RC, Hanson RL. Core Clock Gene Expression in Muscle and Adipose Tissue is Correlated with Metabolic traits. Poster session: American Diabetes Association 73rd Scientific Sessions; 2013 June 20-25; Chicago, IL.
6. Allie Evans, **Brian Grice**, Steven Horbal, Raquel Zemtsov Lauren Wright, Josselyn Howell, Andrea Pfeifle, Javier Sevilla-Martir, Aurelian Bidulescu. Trial to Improve Inter-professional Practice Behaviors at a Student-Run, Free Clinic. Jefferson Center for Interprofessional Education (JCIPE) conference, Interprofessional Care for the 21st Century: Redefining Education and Practice; 2016

7. Allie Evans, **Brian Grice**, Steven Horbal, Aurelian Bidulescu, Andrea Pfeifle. Increasing Interprofessional Practice Behaviors to Enhance Knowledge and Improve Quality of Experience for Students. Learning Together at the Nexus: National Center Summit on the Future of IPE Nexus Fair; 2016.
8. **Brian A Grice**, Kelly J. Barton, Jeffrey S. Elmendorf. Skeletal Muscle Membrane Cholesterol Accumulation and Early Insulin Resistance in Mice Fed a High-Fat Diet. Oral Presentation Indiana Physiologic Society; 2016.
9. **Brian A Grice**, Kelly J. Barton, Jeffrey S. Elmendorf. Skeletal Muscle Membrane Cholesterol Accumulation and Early Insulin Resistance in Mice Fed a High-Fat Diet. American Diabetes Association; 2016.
10. **Brian A Grice**, Lixuan Tackett, Jeffrey S. Elmendorf Membrane Cholesterol Accumulation is a Causal Mechanism of Skeletal Muscle Insulin Resistance Mediated by Hexosamine Biosynthesis Pathway Post-Translational Modifications to SP1. American Diabetes Association; 2019.

Letter

1. **Grice, Brian**. 2012. "The Celebrity Game (1 Letter)." The New York Times, March 19, sec. Science. http://www.nytimes.com/2012/03/20/science/the-celebrity-game-1-letter.html

Projects

Ongoing

1. The role of intracellular cholesterol in the development of diabetes and insulin resistance.
2. Data analysis methods in research.

Completed

1. Trial to improve inter-professional practice behaviors at a student run clinic.
2. Brain-derived neurotrophic factor in human subjects with function-altering melanocortin-4 receptor variants.
3. The relationship between insulin sensitivity and maximal oxygen uptake is confounded by method of adjustment for body composition.
4. SAS programs to monitor QC/QA programs for clinical laboratory equipment.
5. Comparison of disposition index and its components, insulin secretion and sensitivity, as predictors of type 2 diabetes.
6. Long-term Mortality Outcomes of Women with Type 2 Diabetes During Pregnancy.
7. Lifestyle Intervention for Expectant Moms (LIFE-Moms) Clinical Trial
8. Persistent Organic Pollutants and Risk of Diabetes.